The Role of Phospholipase C Like 1 in The Maintenance of Uterine Quiescence During Human Pregnancy

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Declaration

This thesis is submitted to the University of Warwick in support of my application for the degree of Doctor of Philosophy. It has been composed by myself and has not been submitted in any previous application for any degree. All the experiments have been conducted and analysed by the author except for:

i) 6.2.2- Identifying what region within the promoter of the PLCL-1 locus undergoes increased chromatin accessibility upon progesterone treatment . This experiment was conducted and analysed by Dr Raffaella Lucciola.

ii) 4.2.3 - Bioinformatics analysis on the results obtained from RNA sequencing was done by Dr Tauqeer Alam.

iii) 3.2.1 - The western blotting was done by Dr Paul Brighton.

Abstract

With the rising rates of preterm birth, an in-depth understanding of the molecular mechanisms that govern preterm labour and uterine quiescence is needed, as this may lead to identifying drug targets that can prevent preterm labour or lead to reliable tests that identify those are at risk for premature labour. This thesis, focuses on Phospholipase C like-1 (PLCL-1). PLCL-1 is a protein that is similar in structure to PLC-δ1, however PLCL-1 is catalytically inactive. PLCL-1 has been shown to be down-regulated in the myometrium of woman that are in labour compared to those that are not, this suggests a possible role for PLCL-1 in uterine quiescence and exploring the role PLCL-1 plays in uterine quiescence might explain one molecular pathway that leads to parturition. Calcium imaging disclosed that PLCL-1 reduced IP_3 mediated calcium release in primary myometrial cells. Western blotting and RT-PCR illustrated that PLCL-1 transcription is induced by progesterone, this indicates that PLCL-1 is a progesterone responsive gene. High-throughput RNA sequencing showed that a loss in PLCL-1 resulted in an increase in phosphodiesterases, this led to a reduction in the levels of the pro-quiescent molecule: cAMP. In conclusion, this thesis demonstrates that PLCL-1 is downregulated in the labouring myometrium because it functions as a pro-quiescent protein, and its loss might lead to an increase in contractility.

Abbreviations

$[Ca2+]_i$	Intracellular calcium concentration
20α-HSD	20-α-hydroxysteroid dehydrogenase
AC	Adenylyl cylase
ANP	Atrial natriuretic peptide
AP	Action potential
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
cAMP	Cyclic adenosine monophosphate
CAPs	Contraction associated proteins
cGMP	Cyclic guanosine monophosphate
CGRIP	Calcitonin gene-related peptide
COX	Cyclo-oxygenase
CRH	Corticotropin-releasing hormone
CRH-BP	CRH binding protein
CS	Calcium sensitisation
CX43	Connexin 43
DAG	Diacylglycerol
DHEA-S	Dehydroepiandrosterone sulphate
E2	Estradiol
E3	Estriol
eNOS	Endothelial nitric oxide synthase

ERE	Oestrogen response elements
ET-1	Endothelin 1
fFN	Foetal fibronectin
FSH	Follicle-stimulating hormone
GC	Guanylyl cyclase
GPCR	G protein coupled receptor
HA	Hydrophilic glycosaminoglycan
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HPA	Hypothalamic pituitary axis
IL	In labour
IP_3	Inositol trisphosphate
LH	Luteinising hormone
MLC-20	Myosin light chain- 20kDa
MLCK	Myosin light chain kinase
MLCP	Myosin light chain phosphatase
MSMC	Myometrial smooth muscle cell
mTOR	Mammalian target of rapamycin
NIL	Not in labour
NO	Nitric oxide
NSCC	Nonselective cation channels
OT	Oxytocin
OTR	Oxytocin receptor

P4	Progesterone
PDE	Phosphodiesterase
PGDH	Prostaglandin dehydrogenase
PGF2α	Prostaglandin F2α
PKA	Protein kinase A
PKG	Protein Kinase G
PLC	Phospholipase C
PLCL-1	Phospholipase C like 1
PP1	Protein Phosphatase 1
PP2A	Protein Phosphatase 2A
PPROM	Preterm premature rupture of membranes
PR	Progesterone receptor
PSF	Protein-associated splicing factor
ROCK	Rho-associated protein kinase
SMCs	Smooth muscle cells
VOCC	Voltage operated calcium channel
TPM	Transcripts per million
WHO	World Health Organisation
ZIPK	Zipper-interacting protein kinase

Chapter 1

Introduction

1.1- Preterm Birth: Global Rates, Outcomes, Risk Factors and Current Treatments

The number of preterm births worldwide is estimated to be around 15 million annually. The rate of preterm birth for each country varies between 5% and 18%, in the UK 7.2% of all births are classified as preterm (Blencowe *et al*, 2012) (Blencowe *et al*, 2013) (Office of National Statistics, 2018) (WHO, 2018). The World Health Organisation (WHO) defines preterm birth as any birth that occurs before the 37 weeks of gestation and preterm birth is subcategorised subject to gestational age (WHO, 1977). Births that occur between weeks 32 to 37 are categorised as moderately preterm, and those between 28 to 32 weeks are considered as very preterm. Any birth before the 28th week of gestation is categorised as extremely preterm. Preterm birth is the primary cause of perinatal mortality; in the UK 50% of neonatal deaths are attributed to prematurity (Liu *et al,*2016) (Blencowe *et al*, 2013) (Office of National Statistics, 2018). The likelihood of survival decreases the earlier the baby is delivered (Saigal and Doyle, 2008) (Moser *et al*, 2008) (Costeloe *et al,* 2012). Premature neonates who do survive have a greater risk of suffering from morbidities. Preterm birth is associated with a higher risk of motor disability, cognitive impairment, vision and hearing impairments, periventricular leukomalacia, sepsis and respiratory distress syndrome (Marlow *et al*, 2005) (Blencowe *et al*, 2013) (Petrini *et al*, 2009) (Bolisetty *et al*, 2014) (Pierson *et al*, 2004) (Stoll *et al*, 2002).

30% of preterm births are medically indicated due to maternal or foetal distress, 25% are caused by premature preterm rupture of the membranes (PPROM) and 45% are

idiopathic. There are a few associated risk factors for either PPROM or idiopathic preterm birth and they include: a previous history of preterm birth or cervical surgery, multiple pregnancy, race, low body mass index, periodontal disease, vaginal infection, tobacco or alcohol use and socio-economic status (Figure 1.1) (Goldenberg *et al*, 2008).

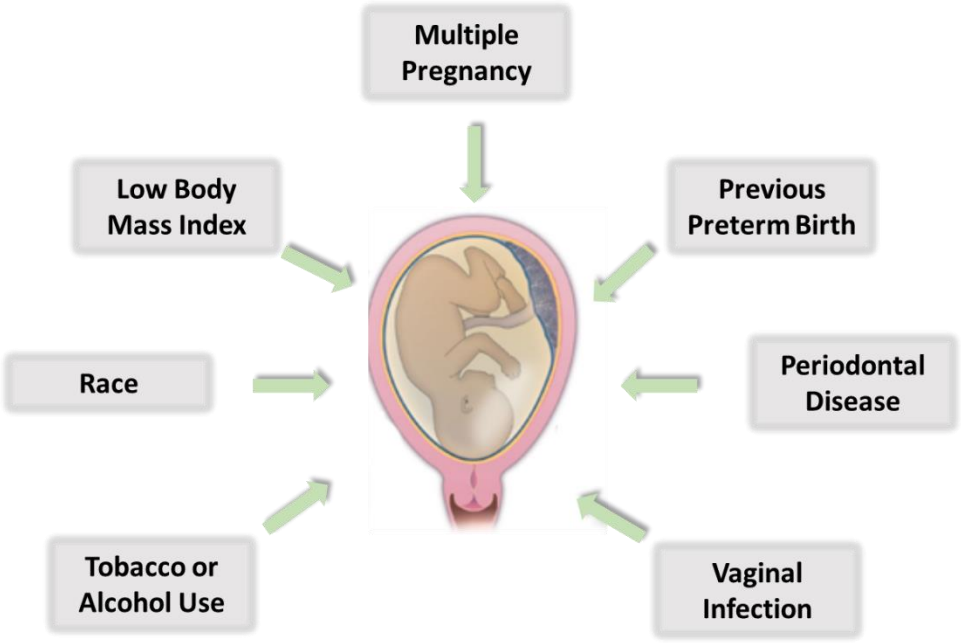


Figure 1.1- ***Risk factors of Preterm Birth****.* The risk factors associated with preterm birth are both environmental and genetic. Figure adapted from Romero *et al*, 2014.

Clinicians rely on transvaginal sonographic cervical length (CL) and cervicovaginal foetal fibronectin (fFN) measurements, to predict the likelihood of a woman going into preterm birth. Foetal fibronectin is present in the amniotic fluid and placenta and damage to foetal membranes results in the release of fFN into the cervix and

vagina, which can then be measured as a biochemical marker for preterm birth (Iams *et al*, 1996) (Matsuura *et al*, 1985) (Lockwood *et al*, 1991). However, both methods have a low positive predictive rate, 21% for CL and 17% for fFN (Arisoy and Yayla, 2012).

Tocolytics are drugs that are used to delay the onset of labour when preterm birth has been diagnosed, this allows for transfer of the woman to receive tertiary care and antenatal corticosteroids to improve foetal lung function (Goldenberg, 2002) (Liggins *et al*, 1972) (Roberts *et al*, 2017). Tocolytics are grouped depending on their target and mode of action and they include: betamimetics, calcium channel blockers, oxytocin receptor blockers, prostaglandin inhibitors, nitrates and magnesium sulphate. Although some tocolytics can delay the onset of labour, there is no clear evidence that they reduce neonatal mortality and morbidity rates. Therefore, there is a need for more reliable diagnostic tests for preterm births and for more effective tocolytics (Anotayanonth *et al*, 2004) (King *et al*, 2003) (Papatsonis *et al*, 2005) (King *et al*, 2005) (Crowther *et al*, 2006).

1.2 - Uterine Anatomy and Myometrial Function During Pregnancy

The uterine corpus (main body) is made up of three walls: an inner mucosal layer that lines the uterine cavity (endometrium), a middle smooth muscle layer (myometrium) and a thin outer layer called the perimetrium. The myometrium is separated into three poorly demarcated layers: the stratum supravasculare that is adjacent to the perimetrium, which is adjacent to the extremely vascularised stratum

vasculare, which is adjacent to the stratum subvasculare (Blanks *et al*, 2007). The myometrium is made up of myometrial smooth muscle cells (MSMCs), which are arranged into ~300µm ±100µm diameter bundles, each bundle is separated by connective tissue and microvasculature. The bundles are then arranged into 1–2 mm diameter cylindric structures called fasciculi, which are surrounded by a dense collagen matrix and blood vessels (Figure 1.2) (Goetller, 1968).

Throughout pregnancy, the myometrium is in a non-contractile (quiescent) state to allow for full in-utero development of the foetus until term is reached. Whilst in the quiescent state, the myometrium undergoes physiological remodelling, via hyperplasia and hypertrophy in preparation for powerful uterine contraction to support parturition (Douglas *et al*, 1988) (Reynolds *et al,* 1992) (Shynlova *et al*, 2007) (Jaffer *et al*, 2009). The spatial orientation of the muscle fibres of the myometrium produces a directional force to aid the expulsion of the foetus from the uterus and through the cervix and vagina. Weiss *et al* and Lutton *et al* both describe circular muscle fibres in the stratum subvaculare near the uterine cavity and longitudinal muscle fibres near the cervix. Whilst the rest of the myometrium was found to be arranged in a disorderly manner with different fibres in erratic orientations (Weiss *et al*, 2006) (Lutton *et al*, 2017).

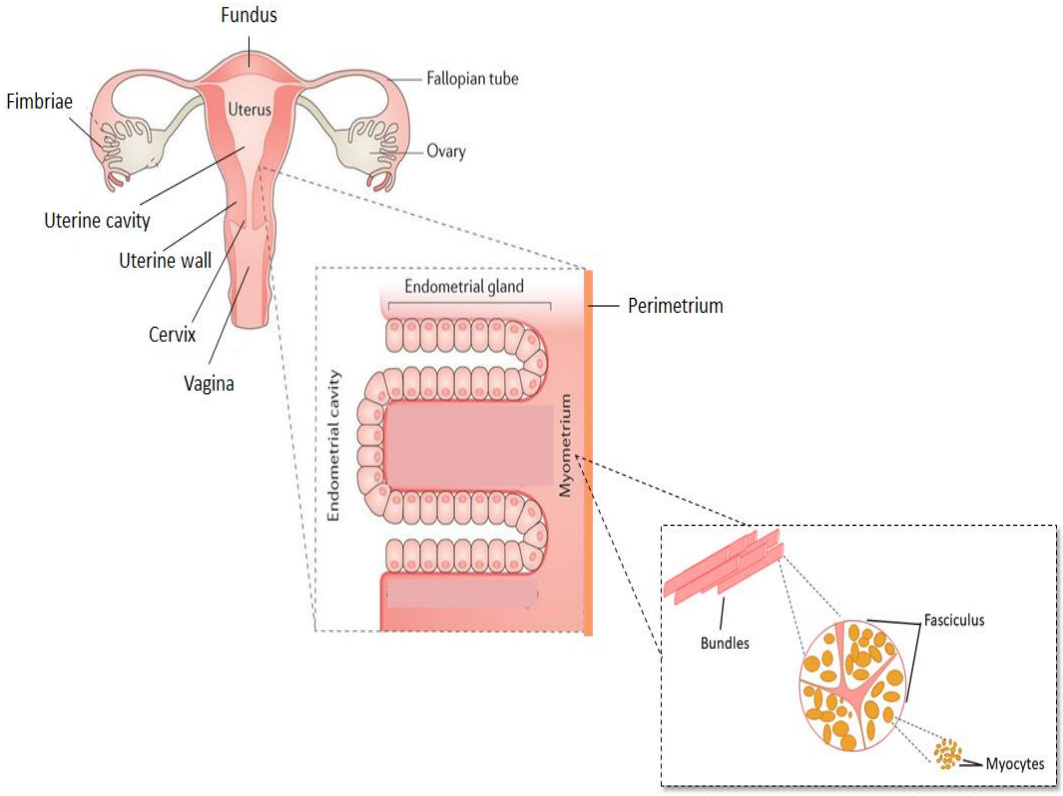


Figure 1.2- ***Structure of the Uterus and Myometrium.*** The uterus wall is made up of the endometrium (facing the uterine cavity), myometrium (middle layer) and perimetrium (outer layer). The myometrium is made up of bundles which are arranged in cylindric structure called fasciculus. The basic unit of the myometrium is the smooth muscle cell (myocyte). Figure adapted from Karnezis *et al*, 2017.

1.3- Molecular Basis of Contraction

The key basis for contraction in the MSMC is the cyclic cross-bridge formation between myosin and actin filaments. The 20kDa Myosin light chain (MLC-20) attaches and detaches from the actin filaments; attachment causes shortening (contraction) of the MSMC and detachment causes relaxation. The cross-bridge formation is activated by myosin light chain kinase (MLCK), which phosphorylates the

globular head of MLC-20, thereby activating myosin ATPase activity to allow for ATP hydrolysis (Rayment *et al*, 1993) (Ishijima *et al*, 1998) (Figure 1.3). ATP hydrolysis provides the energy needed for MLC-20 to bind to actin, causing actin filament sliding, leading to shortening of the MSMC i.e. contraction (Word *et al*, 1993).

A rise in intracellular calcium concentration $[Ca^{2+}]_i$ leads to the activation of MLCK. Activation of MLCK is crucial for contraction to occur in the myometrium. Inhibition of MLCK using wortmannin and ML-9, inhibits myometrium contraction (Longbottom *et al*, 2000). Initially 4 calcium ions bind to calmodulin, which has two EF hand Ca^{2+} binding sites in its N-terminus and another two EF hand Ca^{2+} binding sites in its C-terminus. When the calcium ions bind to calmodulin, it undergoes a conformational change, which causes the exposure of a hydrophobic pocket (Johnson *et al*, 1996) (LaPorte *et al*, 1980). The activated calmodulin bound to 4 calcium ions can then activate MLCK. Action potential (AP) firing on MSMC plasma membrane leads to the opening of the voltage operated calcium channels (VOCC). The AP mediated calcium influx leading to contraction is called excitation-contraction coupling.

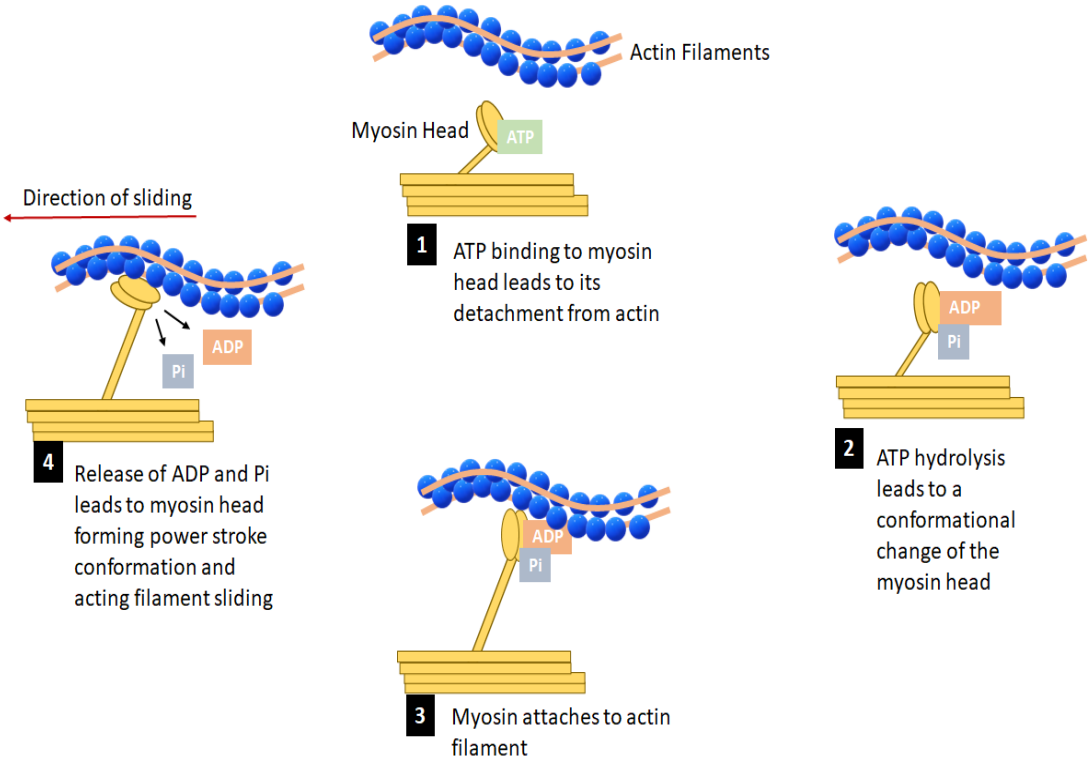


Figure 1.3 - ***Actin-myosin Cross Bridge Cycling in the Smooth Muscle Cell.***

1.3.1- Excitation-Contraction Coupling

The myometrium displays spontaneous electrical behaviour without any stimuli from the central nervous system or from hormones. There has been no definitive proof of any pacemaker cells within the myometrium, although a pacemaker region in the maternal-placental interface of the rat myometrium has been recently identified (Lutton *et al*, 2018). MSMCs maintain a concentration gradient of ions: (Cl^-, Ca^{2+}, K^+ and Na^+) across the plasma membrane. MSMC maintain the concentration gradient by the active transport of ions using ion pumps and transporters present on the

plasma membrane. The intracellular resting membrane potential of MSMCs is around -55mV. Action potential firing requires cellular depolarisation to reach threshold potential. The opening of ion channels on the plasma membrane allows the movement of ions down their electrochemical gradient. Action potential firing in the MSMC is due the opening of calcium channels, which causes the inward flow of calcium into the MSMC through L type VOCC (L-VOCC) (Shmigol *et al,* 1998). The human myometrium also expresses T-type VOCC (T-VOCC), and inhibiting T-VOCC using nickel reduces the contraction frequency (Blanks *et al*, 2007). Repolarisation of the MSMC is instigated by the opening of potassium channels leading to an efflux of potassium ions, this leads to a loss of positively charged potassium ions, leading to the repolarisation of the cell (Mironneau, 1973)(Inoue *et al*, 1990) (Figure 1.4).

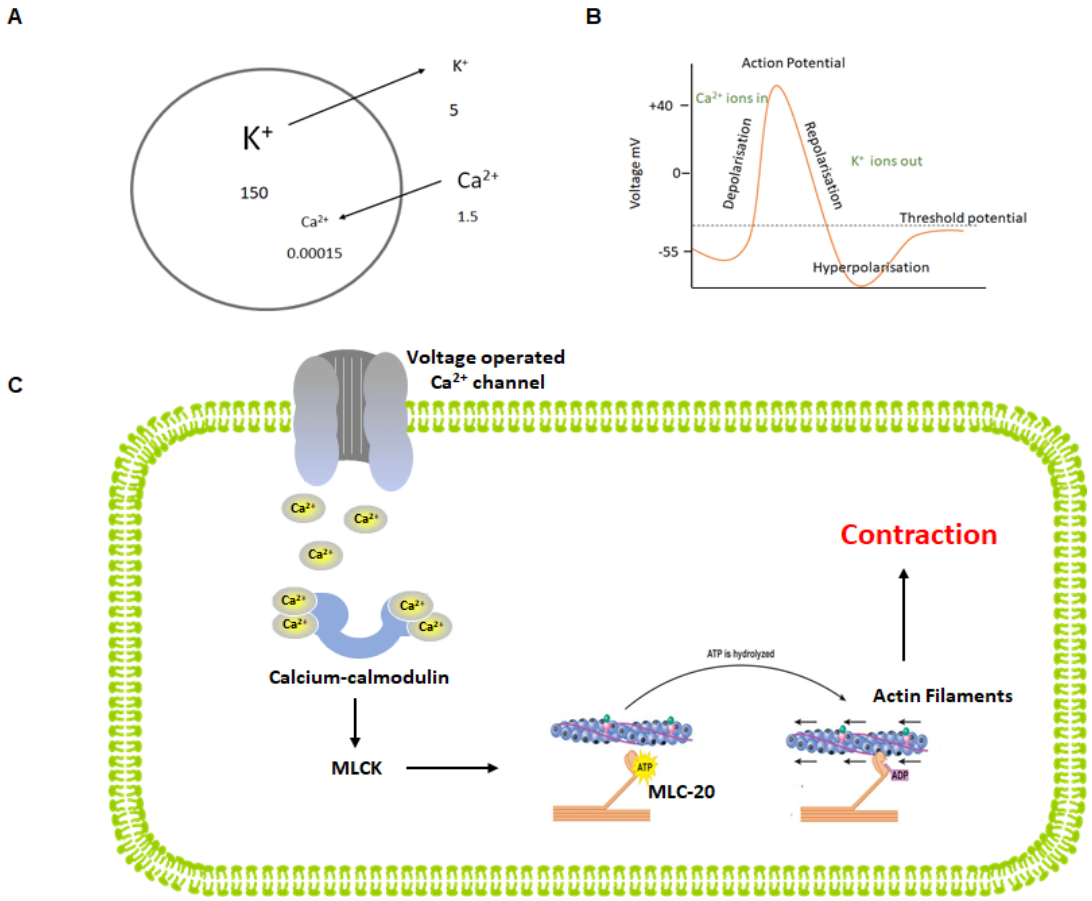


Figure 1.4- ***Excitation-Contraction Coupling***. A) The electrochemical gradients of potassium and calcium ions present on the MSMC plasma membrane. Potassium ions have higher concentration (mmol/L) inside the cell, and will flow outwards, Calcium ions have higher concentration outside the cell and will flow inwards. B) The basis of an action potential in the MSMC. The flow of calcium ions into the cells causes depolarisation and action potential firing, the flow of potassium ions out the cells causes repolarisation. C) Excitation of MSMC activates MLCK, which phosphorylates myosin light chain (MLC-20) causing ATP hydrolysis, binding of myosin head to actin and actin filament sliding (contraction).

1.4 - Phases of Pregnancy and Parturition

Pregnancy and parturition can be divided into 5 different phases. These phases are: i) implantation of the blastocyst, and placentation ii) myometrial quiescence to allow for foetal growth and development iii) activation of molecular pathways that promote uterine contractility iv) stimulation, which involves cervical ripening and active labour v) involution, whereby the uterus reverts to a non-pregnant condition (Rubens *et al*, 2014).

During the quiescent phase of pregnancy, the uterus is dominated by pro-quiescent factors such as progesterone (P4), nitric oxide (NO), cyclic AMP (cAMP) and cyclic GMP (cGMP). There is a loss in pro-quiescent factors during the activation and stimulation phase and there is upregulation and activation of contraction associated proteins (CAPs) such as: oxytocin (OT), oxytocin receptor (OTR), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and connexin 43 (Cx43) (Figure 1.5) (Smith, 2007) (Romero *et al*, 2014) (Rubens *et al*, 2014).

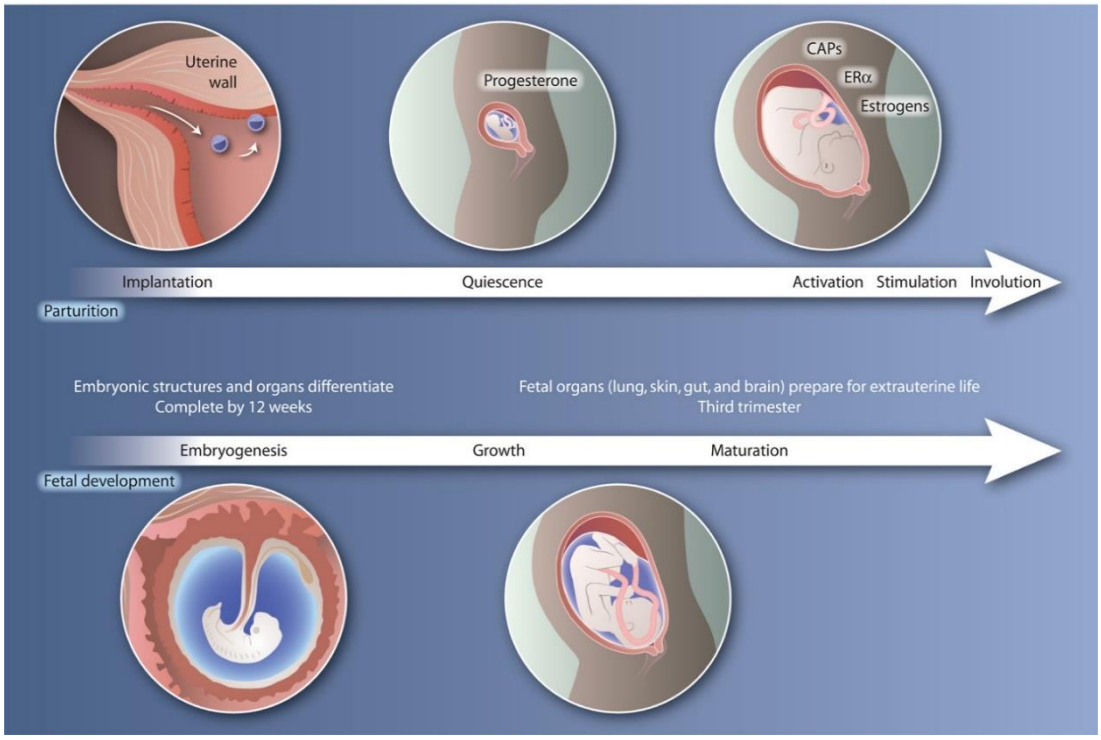


Figure 1.5 - ***Phases of Pregnancy and Parturition.*** The uterus is in a quiescent state for majority of the pregnancy to allow for embryogenesis and foetal maturation. Progesterone and other pro-quiescent factors maintain the uterus in the non-contractile phase. At the end of the third trimester there is upregulation of contraction associated proteins (CAPs) and oestrogen in preparation for parturition. Figure from Rubens *et al,* 2014.

1.5- The Quiescence Phase of Pregnancy

1.5.1- The Role of Cyclic Nucleotides in Maintaining Uterine Quiescence.

Cyclic nucleotides: cAMP and cGMP play an important role in minimising the effects of calcium in MSMC and keeping the myometrium quiescent. Cyclic AMP is synthesised from ATP by adenylate cyclase (AC), which is a transmembrane protein, with 12 membrane spanning motifs. The highly conserved and hydrophobic catalytic

core of AC is made of up of two regions: $C1_a$ and $C2_a$ and can bind both $G_{\alpha s}$ and ATP (Tesmer *et al,* 1997). AC is activated by $G_{\alpha s}$, which upon binding causes a conformational change in AC, allowing it to synthesise cAMP from ATP. There are 9 isoforms of AC and all were found to be expressed in the human myometrium (Price *et al,* 2000). All AC isoforms are activated by $G_{\alpha s}$, however other modulators have opposing effects on different isoforms. For example, Protein Kinase C (PKC) inhibits AC9 but can activate AC2, AC4, AC5 and AC7 (Cumbay and Watts, 2004) (Halls and Cooper, 2011). B-adrenergic agonist such as relaxin and prostacyclin maintain uterine quiescence by activating AC mediated synthesis of cAMP (Challis *et al*, 2000) (Norwitz, 1997).

Protein Kinase A (PKA) is one the effector proteins of cAMP. PKA is a tetrameric protein and has two regulatory subunits (R) and two catalytic subunits (C). Two cAMP molecules bind to the two R subunits, causing the catalytic units to detach from the R subunits. The catalytic units are then free to phosphorylate their substrates (Tillo *et al*, 2017).

One of the substrates of PKA is MLCK. Phosphorylation of MLCK by PKA, causes MLCK to lose its affinity for calcium-calmodulin, thus PKA inhibits MLCK, leading to smooth muscle cells (SMCs) relaxation. PKA expression decreases in pregnant human myometrium, during the latter stages of gestation (Ku *et al*, 2005). There is conflicting evidence on the effect of cAMP/PKA pathway on L type VOCC in SMCs. Activation of the cAMP/PKA has been shown to inhibit VOCC (Liu *et al*, 1997), it has

also been shown to activate VOCC (Ruiz-Velasco et al, 1998). PKA phosphorylates and enhances the BK_{Ca} channel activity in pregnant rat myometrium, causing an outward flow of K^+ ions, which inhibits AP firing and excitation-contraction coupling (Perez and Toro, 1994). PKA phosphorylates and inhibits $PLC\beta_3$, thus PKA is able to inhibit $G_{\alpha q}$ mediated calcium release (Yue *et al*, 1998).

Additionally, cAMP/PKA maintain quiescence in the uterus by inhibiting calcium sensitisation (CS). The effects of calcium in the MSMC can be amplified, without the need for a rise in calcium concentration and therefore increase contractile force; this is a process called calcium sensitisation (CS). Calcium sensitisation is thought to depend on the phosphorylation state of myosin light chain (Somlyo and Somlyo, 2003). Myosin light chain phosphatase (MLCP) is a protein that dephosphorylates myosin light chain and inhibits contraction(Somlyo and Somlyo, 1994). CS in SMCs is regulated by the small GTPase rhoA. $G_{\alpha q11}$ and $G_{\alpha q12/13}$ activate rhoA, which in turn activates Rho kinase (ROCK), which phosphorylates the PP_{1C} catalytic subunit of MLCP, this inhibits MLCP (Hirata et al, 1992) (Gong et al, 2001). Following the inhibition of MLCP myosin light chain is able to stay in an active, phosphorylated, state (Noda *et al*, 1995). There is an up-regulation of Rho kinase mRNA during pregnancy, and this indicates heightened CS in the myometrium with increasing gestational duration (Moore *et al,* 2000). However, during the quiescence phase of pregnancy CS is inhibited by the actions of PKA.

PKA inhibits calcium sensitisation by phosphorylating rhoA, causing rhoA to translocate from the membrane to the cytosol and preventing the activation of ROCK (Murthy *et al*, 2003). PKA also inhibits $G_{\alpha q13}$ by phosphorylation, which blocks $G_{\alpha q13}$ mediated activation of rhoA (Manganello *et al*, 2003) (Figure 1.6).

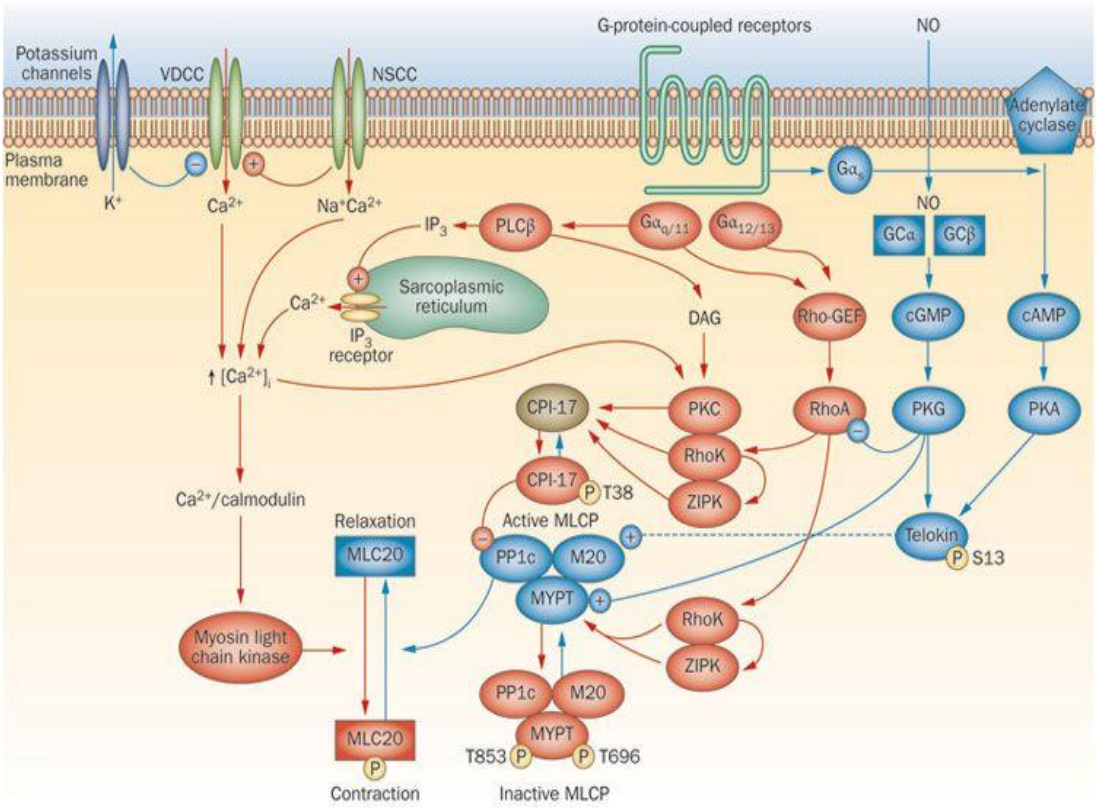


Figure 1.6 - ***Major Cellular Mechanisms Controlling Contraction and Quiescence in SMCs.*** Pathways leading to contraction are shown in red, and pathways linked to relaxation are shown in blue. Abbreviations: DAG, diacylglycerol; IP_3, inositol 1,4,5-trisphosphate; MLC20, 20kDA light chain of myosin; MLCP, myosin light chain phosphatase; NO, nitric oxide; NSCC, nonselective cation channels; PKA, cAMP-dependent protein kinase; PKG, cGMP-dependent protein kinase; PLCβ, phospholipase Cβ; VDCC, voltage-dependent Ca^{2+} channels; ZIPK, zipper-interacting protein kinase. Figure from Sanders *et al,* 2012.

Heat shock proteins (HSPs) are synthesised as a reaction to any physical cellular stresses such as heat. Some HSPs are able to prevent actin polymerisation and thus inhibit contraction (Mounier and Arrigo, 2002) . Heat shock protein 20 (HSP20) is a substrate of PKA. Phosphorylated HSP20 has been found to induce vascular smooth muscle relaxation (Woodrum *et al*, 2003). In MSMCs, PKA phosphorylates HSP20, which then co-localises with α-actin, this illustrates a mechanism for myometrial relaxation (Tyson *et al*, 2008).

Another substrate of PKA is the human ether-a-go-go-related gene (hERG) potassium channel. PKA phosphorylation increases hERG protein abundance (Chen *et al*, 2009). The hERG channel subdues myometrial contractile amplitude and duration prior to labour and its activity is reduced during labour. Therefore cAMP/PKA promotes uterine quiescence by increasing the activity of the hERG channel (Parkington *et al*, 2014).

Endothelial nitric oxide synthase (eNOS) is found in MSMC and synthesises nitric oxide from L-arginine (Khorram *et al*, 1999). One of the targets of nitric oxide is guanylyl cyclase, which synthesises cGMP from GTP. Both NO and cGMP caused uterine relaxation in pregnant rats (Yallampalli *et al*, 1994). In human MSMC; cGMP blocked oxytocin induced calcium release (Buxton, 2009).

1.5.2- Progesterone: Synthesis, Mechanism of Action and its Pro-quiescent Functions

Progesterone is a steroid hormone that plays a functional role in a wide variety of biological processes such as in the immune system, reproductive system and neuronal activity. In reproductive health, progesterone's main function is in the decidualisation of the endometrium and keeping the uterus in a non-contractile state during pregnancy. Progesterone is produced by the adrenal gland, corpus luteum, ovaries and the placenta (Gellersen *et al*, 2009). In the inner membrane of the mitochondria, cholesterol is first converted to pregnenolone by cytochrome P450scc, thereafter pregnenolone is synthesised into progesterone by 3β-hydroxysteroid dehydrogenase (Figure 1.7) (Schumacher *et al*, 2001).

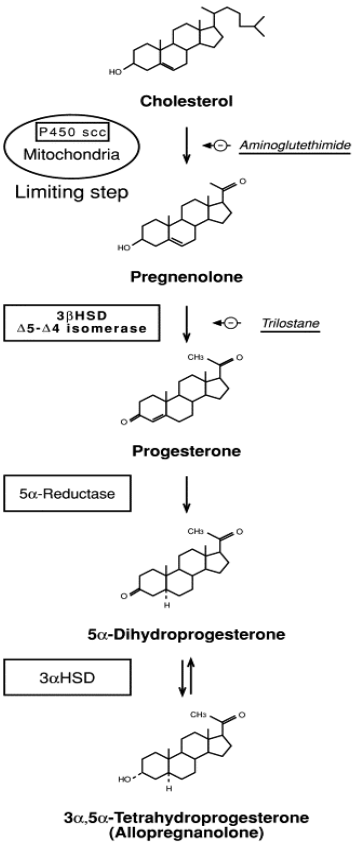


Figure 1.7-*Progesterone Synthesis*. Figure from Schumacher *et al*, 2001.

Steroidogenic cells secrete biologically active progesterone into the blood stream. Corticosteroid-binding globulin binds to secreted progesterone with a high affinity, hence it's able to regulate the concentration of free progesterone that is available for progesterone sensitive cells (Hammond, 2016). Unbound progesterone diffuses across the plasma membrane and binds to progesterone receptors. There are two known isoforms of the progesterone receptor: PRA and PRB, which are transcribed from the same gene that utilises two different promoters (Kastner *et al*, 1990). PRA and PRB regulate different biological processes. For example, a lack of PRB causes aberrations in mammary gland development. Whilst PRA null mice were not able to ovulate, undergo decidualisation and were infertile (Mulac-Jericevic *et al*, 2003) (Mulac-Jericevic *et al*, 2000).

PRA and PRB have a very similar structure; they both have a ligand binding domain (LBD), a DNA binding domain (DBD) and an N-terminus domain (NTD) (Figure 1.8). PRB has an extended NTD and is a longer than PRA by 164 amino acids. PRA has two regions termed transcriptional activation functions (AF1 and AF2), the two regions can operate independently or synergistically. PRB has an extra transcriptional activation function (AF3) in the extended part of the NTD (Sartorius *et al*, 1994). The presence of AF3 in PRB is attributed to the difference in transcriptional activity between the two progesterone receptor isoforms (Dong *et al*, 2004). PRB is a stronger transcriptional activator than PRA. The synergism between AF1, AF2 and AF3 that is not present in PRA makes PRB a stronger transcriptional activator. Loss of AF3 region in PRB significantly reduces the transcriptional activity of PRB (Tung *et al*,

2006). In addition to the structural differences between the progesterone receptors, PRA supresses PRB functional activity (Vegeto *et al*, 1993).

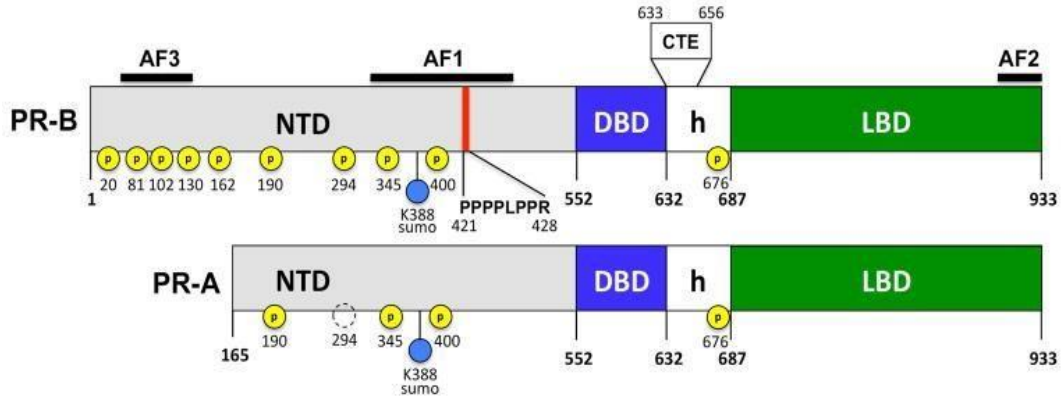


Figure 1.8- ***Schematic Representation, Showing the Structural Differences of Progesterone Receptor A (PR-A) and Progesterone Receptor B (PR-B).*** Abbreviations: N-terminus domain (NTD), DNA binding domain (DBD), ligand binding domain (LBD), p (phosphorylation sites), h (hinge domain), AF (activation functions). Figure from Hill *et al,* 2012

Binding of progesterone to PR, causes PR to dimerise and undergo a conformational change, and disassociate from regulatory proteins (Smith *et al*, 1990). PRA can dimerise as homodimers or heterodimers (Hill *et al*, 2012). During the secretory phase of the human menstrual cycle, progesterone binding to PR causes the receptor to translocate to nuclear aggregates (Arnett- Mansfield *et al*, 2007). After progesterone binding to PR, it binds to progesterone response elements on target genes. PR recruits co-regulators that help to either activate or repress transcription of various genes. There are over 300 co-regulators of PR (Scarpin *et al*, 2009). The functional differences between PRA and PRB can be partially attributed to differential

recruitment of co-regulators to the two progesterone receptor isoforms (Giangrande *et al*, 2000).

The putative function of progesterone in the myometrium is to keep the uterus in a non-contractile state, this is to allow for full development of the foetus until the foetus is ready for extrauterine survival. One of the ways progesterone keeps the myometrium in a quiescent form is by regulating ion channel expression on the MSMC membrane.

Potassium channels are important in keeping the membrane potential close to the reversal potential for K^+, making it unlikely for AP firing and subsequent excitation-contraction coupling. The Na/K ATPase pump is important for maintaining the potassium concentration gradient present between the extracellular space and the MSMC cytoplasm. Progesterone upregulates the mRNA encoding the Na/K ATPase β1 subunit of luminal epithelial cells of the mouse uterus (Deng *et al*, 2013). Progesterone upregulates mRNA of BK_{Ca} and the voltage operated potassium channel KCND3 (Shi *et al*, 2015). P4 also upregulates protein levels of other voltage gated potassium channels, such as KCNH1 (Ramirez *et al*, 2013). Additionally, P4 increases the outward current flow from delayed rectifier potassium channels in cultured MSMC, this decreases the likelihood of AP firing and any subsequent excitation-contraction coupling (Knock *et al*, 2001).

In vascular smooth muscle cells progesterone decreases intracellular calcium ion concentration and inhibits L-VOCC inward current (Barbagallo *et al*, 2001). Progesterone also inhibits L-VOCC inward current in gallbladder smooth muscle cells (Wu and Shen, 2010). In striatal neurons, progesterone is known as a potent protector against neurotoxicity caused by excessive calcium concentration, by inhibiting L-VOCC inward current (Luoma *et al*, 2011). Therefore, progesterone can inhibit contraction in SMCs by inhibiting influx of calcium ions through L-VOCC.

Progesterone Increases Expression of Agonists of Uterine Quiescence

Progesterone increases levels of Atrial Natriuretic Peptide (ANP) in primary MSMCs by increasing expression of the serine protease corin. Pro-ANP is the precursor of ANP, and Pro-ANP is converted into ANP by corin (Soloff *et al*, 2011). ANP decreases the frequency of contractions observed in human myometrium (Cootauco *et al*, 2008). ANP activates guanylyl cyclase (GC) and increases cGMP production in myometrium (Carvajal *et al*, 2001). The GC/cGMP/PKG pathway is a well-known pathway that is involved in smooth muscle cell relaxation.

Progesterone increases transcription of Calcitonin gene-related peptide (CGRP) and adrenomedullin in rat myometrium (Thota and Yallampalli, 2005). Both CGRP and adrenomedullin bind to GPCR coupled to $G_{\alpha s}$ subunit which activates adenylyl cyclase. As stated previously, the AC/cAMP/PKA pathway is involved in upholding uterine quiescence. CGRP is upregulated in rat plasma in pregnancy, but is

downregulated during labour (Gangula *et al*, 2000). CGRP decreases contraction in myometrium obtained from pregnant women, this effect is reduced in myometrium obtained from women in active labour. Furthermore, the relaxant effects on the myometrium due to CGRP treatment is lost when a selective CGRP or guanylyl cyclase antagonist is added, this indicates that CGRP regulates both adenylyl cyclase and guanylyl cyclase (Dong *et al*, 1999).

Progesterone Decreases Expression of Agonist of Uterine Contraction

Progesterone reduces expression of endothelin 1 in human MSMCs (Soloff *et al*, 2011). Endothelin 1 (ET-1) is a well characterised and effective vasoconstrictor peptide. Concentrations of ET-1 in rat myometrium increase with increasing gestation time, with the highest concentration measured during the early postpartum period (Kajihara *et al*, 1996). ET-1 binds to ET receptors on MSMC plasma membrane and causes contractions in the rat uterus, by opening VOCC (Kozuka *et al*, 1989). ET receptors are GPCR coupled to $G_{\alpha q}$ and ET-1 ligand binding causes activation of phospholipase C (PLC) and release of intercellular calcium stores and MLC-20 phosphorylation in human myometrial cells (Takuwa *et al*, 1989) (Word *et al*, 1990). Progesterone attenuates ET-1 induced increases in intracellular calcium in human myometrium cells; this may be a model in how progesterone maintains uterine quiescence. Although the mechanism by which progesterone inhibits ET-1 induced rise in intracellular calcium is not known; it could be that progesterone utilises an effector protein (Fomin *et al*, 1999).

Progesterone decreases expression of the CAPS: connexin 43 (Cx43) and cyclooxygenase-2 (COX-2), which are important in the stimulation phase and activation phase respectively (Zhao *et al*, 1996) (Hardy *et al*, 2006).

Progesterone inhibits oxytocin receptor (OTR) transcription in rat myometrium (Soloff *et al*, 1983). Progesterone dependent reduction of OTR expression could be mediated by interleukin 1 (IL-1) signalling. IL-1 recruits NF-κb to the OTR promoter, disrupting the binding of RNA polymerase II to OTR promoter and subsequent OTR transcription (Soloff *et al*, 2006). Progesterone induces IL-1 signalling by upregulating interleukin 1 receptor expression (Soloff *et al*, 2011).

Progesterone also decreases expression of phosphodiesterases 4B and 1C. Phosphodiesterase causes degradation of the pro-quiescent second messengers cAMP and cGMP (Soloff *et al*, 2011).

1.6 The Activation Phase of Pregnancy

1.6.1 –Foetal HPA Axis

The exact mechanism of activation of parturition in humans has not been very well defined, as it has been in other species. For example, in sheep parturition is initiated by activation of the foetal hypothalamic pituitary adrenal axis (HPA) (Liggins *et al*, 1973) (Liggins *et a*l 1967). Sheep with lesions in the foetal hypothalamus undergo

prolonged labour (Gluckman *et al*, 1991). Activation of foetal HPA in sheep causes the secretions of corticosteroids, which activate enzymes 17a hydroxylase and 17–20 lyase; these enzymes then favour the conversion of cholesterol to oestrogen rather than progesterone (Mason *et al*, 1989). The switch in endocrine expression promotes prostaglandin production, cervical softening and increased myometrial contractions (Bernal, 2003) (Beshay *et al*, 2007). Foetal HPA does not play a major role but rather a supportive role in activating parturition in humans, as women who carry an anencephalic foetus have the same gestation length as women who carry a non-anencephalic foetus. Although, there was a huge increase in the variance of the mean delivery dates in anencephalic pregnancies compared to non-anencephalic pregnancies (Honnebier and Swaab, 1973).

1.6.2- Corticotropin-Releasing Hormone (CRH)

The activation phase of pregnancy is characterised by an increase in uterotropins, which upregulate the expression of CAPs, which subsequently cause biochemical and cellular changes that prime the uterus for labour. CAPs stimulate uterine contractility by enhancing actin-myosin cross bridge formation, increasing MSMC excitability and improving cell-to cell connectivity to allow for coordinated contractions (Smith, 2007) (Norwitz, 1999). A key uterotropin during the activation phase is oestrogen. Corticotropin releasing hormone (CRH) stimulates the synthesis of oestrogens. CRH is a 41 amino acid neuropeptide that is secreted by the hypothalamus and the syncytiotrophoblast within the placenta (Vale *et al*, 1981) (Riley *et al*, 1991) (Figure 1.9).

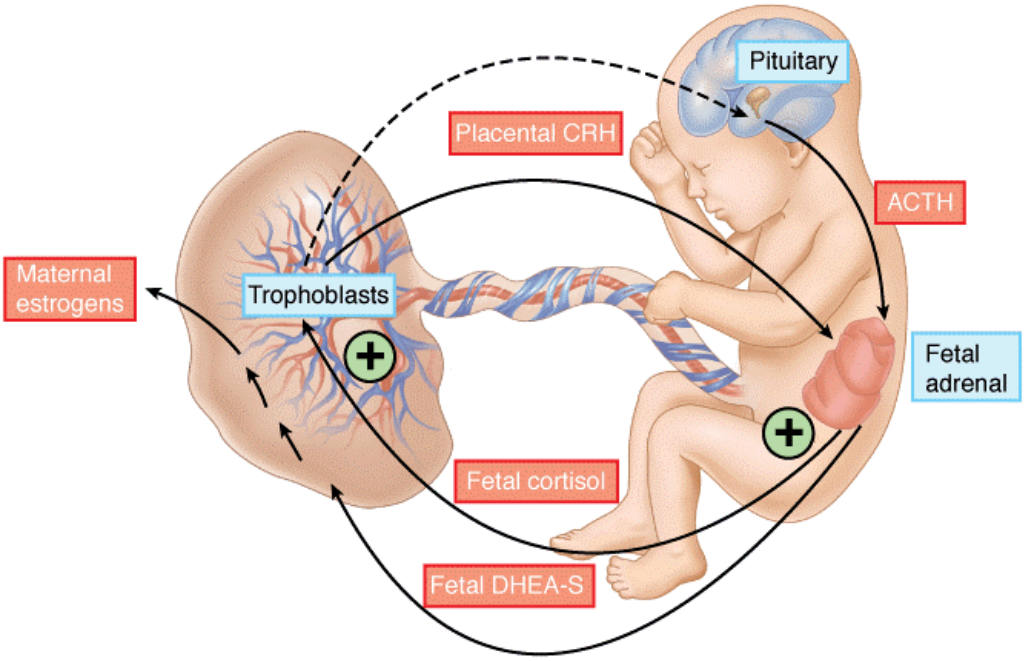


Figure 1.9 – *Foetal Hypothalamic- Pituitary-Adrenal-Placental Axis*. Increase in foetal cortisol levels in the latter stages of pregnancy, stimulates the production of corticotropin-releasing hormone (CRH) from the syncytiotrophoblast in the placenta. CRH stimulates the secretion of adrenocorticotropic hormone (ACTH). Placental CRH also stimulates the production cortisol and dehydroepiandrosterone sulphate (DHEA-S) by the foetal membranes and placenta. Figure from Williams Obstetrics, 23rd Edition, Chapter 6.

The levels of free CRH in maternal system circulation and the foetal fluids increases with increasing gestational age (Sorem *et al*, 1996) (Frim *et al*, 1988). In the early stages of pregnancy, the circulatory levels of free CRH is low as it is bound to its serum binding protein (CRH-BP), in the latter stages of pregnancy CRH-BP levels are reduced in the maternal plasma and the amniotic fluid (Linton *et al*, 1988) (Orth *et al*, 1987). The reduction of CRH-BP in the latter stages increases the levels of free of CRH (Florio

et al, 1997). CRH binds to CRH receptors, which are GPCR that are found in the maternal myometrium and pituitary gland and the foetal membranes, pituitary and adrenal glands. Activation of CRH receptors promotes uterine quiescence for most of the pregnancy, during the latter stages of pregnancy (activation phase) CRH receptor signalling promotes uterine contractility, this is thought to be partly due to changes in CRH receptor subtype expression. Different subtypes of the CRH receptor couple to different G-proteins. There is evidence to suggest that during the quiescence phase of pregnancy CRH activates the $G_{\alpha s}$ and the adenylyl cyclase/cAMP pathway. However, during labour CRH activates the $G_{\alpha q}$ and the PLC/IP_3 pathway, due to differential expression in CRH receptor subtype (Grammatopoulos, 2007) (Beshay *et al*, 2007) (Grammatopoulos *et al*, 1998). CRH receptor signalling promotes the synthesis of dehydroepiandrosterone sulphate (DHEA-S) which is converted into the oestrogens: estradiol (E2) and estriol (E3). Oestrogens bind to nuclear receptors. Activation of E2 receptor on the myometrium leads to increased expression of the CAPs such as: OTR and prostaglandin receptors (Smith *et al*, 1998) (Welsh *et al*, 2012) (Ham *et al*, 1975) (Figure 1.10).

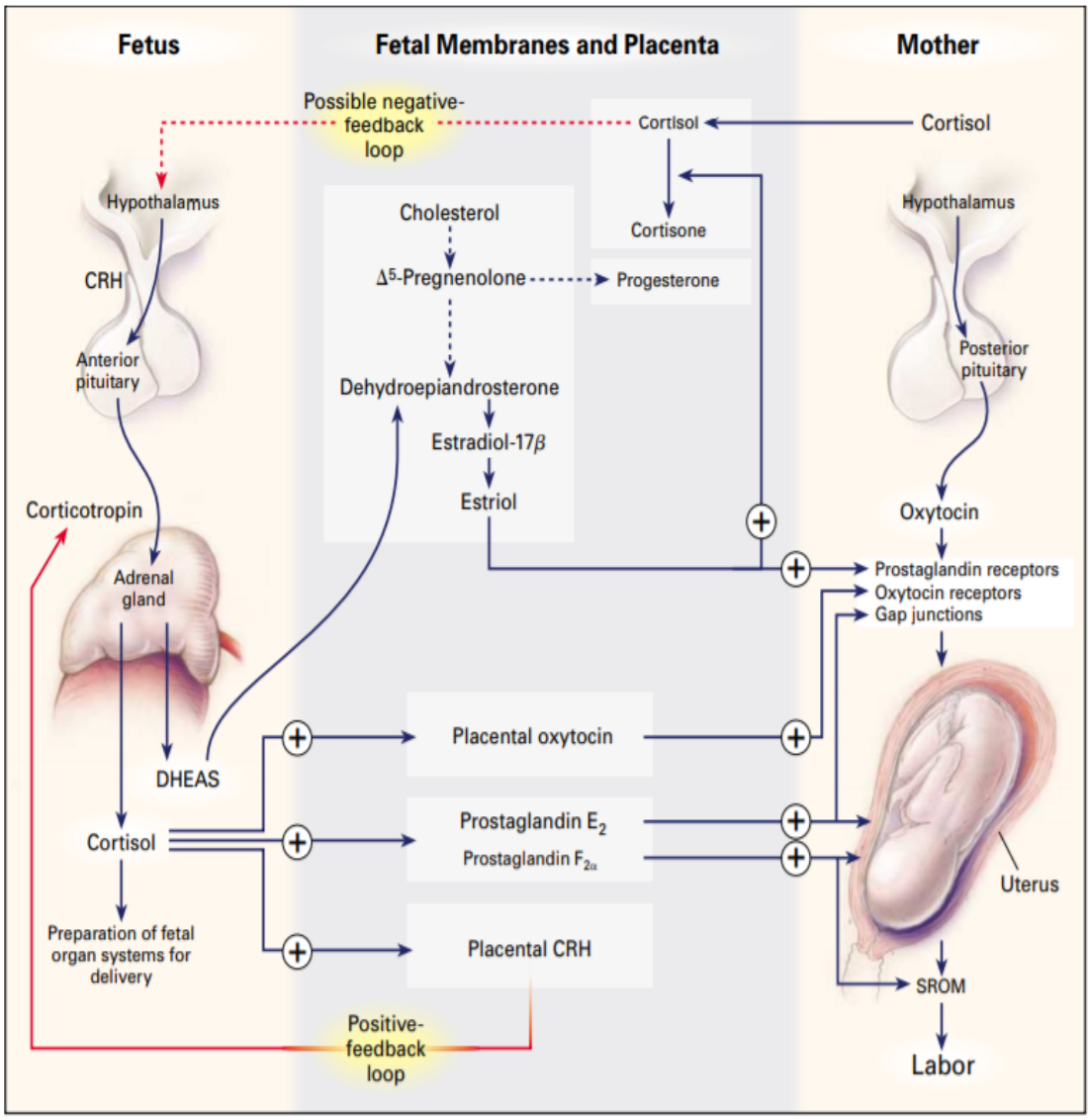


Figure 1.10 - *The Key Hormones and Paracrine and Autocrine Factors Implicated in the Activation Phase*. Corticotropin-releasing hormone (CRH), DHEAS dehydroepiandrosterone sulphate (DHEA-S), and spontaneous rupture of the foetal membranes (SROM). Figure from Norwitz *et al*, 1999.

1.6.2- Gap Junctions

Gap junctions are channels that span the plasma membrane of two cells, to allow for the passage of ions and small molecules from one cell to another (Figure 1.11). The

flow of ions between adjacent cells propagates action potential firing across the myometrium, leading to synchronised contraction during birth (Garfield *et al*, 1977). A gap junction between two adjacent cells is formed when two connexon proteins; one from each cell are merged, which creates a hydrophilic open pore in the plasma membrane. A connexon protein is made up of 6 subunits (connexins) which assemble in a hexameric arrangement. There are 21 different isoforms of connexins and the myometrium expresses four of them: Cx26, Cx40, Cx43 and Cx45 (Garfield *et al,* 1977) (Ou *et al*, 1997) (Sheldon *et al,* 2014).

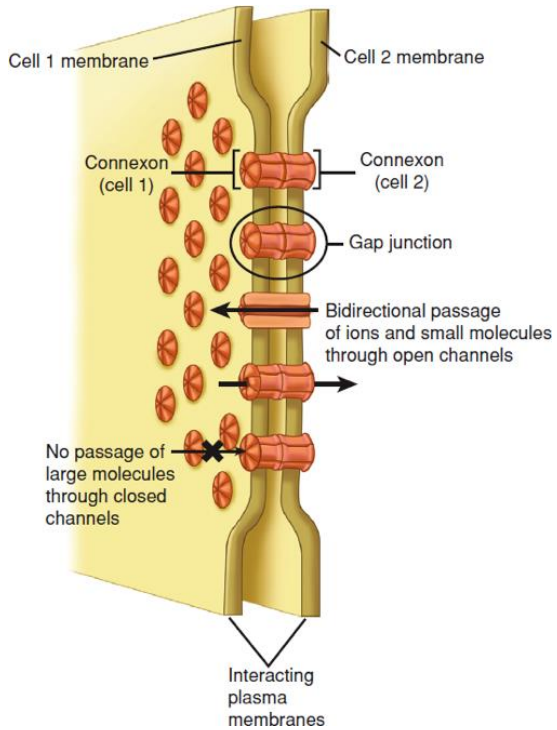


Figure 1.11 - *Schematic Representation of Gap Junctions Between Adjacent Myometrial Cells.* Figure from Williams Obstetrics, 24th Edition, Chapter 21, pg 419

The expression of Cx43 increases with increasing gestational age, with the highest levels found just prior to the onset of labour (Hendrix *et al*, 1992) (Chow and Lye, 1994). Although Cx43 is upregulated in both mice and humans prior to parturition, there is a difference in the mechanism of regulation between the two. In mice, during the quiescence phase Cx43 is directly inhibited by high levels of circulatory progesterone, which acts on PR to recruit the transcriptional co-repressors such as p54NRB, to inhibit expression of Cx43 (Ou *et al*, 1997) (Dong *et al*, 2009). During the activation phase a drop in circulatory progesterone and an increase in oestrogen leads to an increase in Cx43 expression. The promoter for the Cx43 gene in mice contains half palindromic oestrogen response elements (ERE) (Lye *et al*, 1993) (Petrocelli and Lye, 1993) (Kidder and Winterhager, 2015). In humans, PR represses expression of Cx43 by recruiting transcriptional repressors such as polypyrimidine tract binding protein-associated splicing factor (PSF), which recruits histone deacetylases (HDAC) to the Cx43 promoter (Xie *et al*, 2012). In contrast to mice, the human Cx43 gene is upregulated independently of oestrogen receptor by prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) (Geimonen *et al*, 1998) (Xu *et al*, 2013). Treating human MSMCs with indomethacin (prostaglandin inhibitor) reduces expression of Cx43 (Xu *et al*, 2013). Therefore, in humans Cx43 expression is upregulated in the activation phase due to a reduction in PR transcriptional co-repressors (e.g. PSF) and an increase in $PGF_{2\alpha}$ (Kidder and Winterhager, 2015).

The significance of gap junctions for normal labour is illustrated in Cx43 knockout mice. Cx43 null mice experienced delayed labour compared to wildtype mice, however they did deliver suggesting that there is compensation for Cx43 by other connexins (Doring *et al*, 2006). The delayed labour experienced by Cx43 null mice shows the importance of gap junctions in allowing numerous myocytes to contract in concert, which produces the powerful synchronised contractions observed during labour.

1.7- The Stimulation Phase of Parturition

1.7.1- Cervical Ripening

Cervical ripening occurs in the last weeks of gestation, whereby the cervix becomes thin and flexible, which allows for stretching without rupture and the passage of the foetus during labour (Word *et al*, 2007). The structure of the cervix prior to cervical ripening is characterised by condensed highly organised collagen bundles, with hypertrophy and hyperplasia of cervical fibroblast and SMCs. During cervical ripening there is decrease in collagen concentration. The decrease in collagen is attributed to an increase in hydrophilic glycosaminoglycan (HA), non-collagenous proteins and aquaporin water channels (Straach *et al*, 2005) (Buhimschi *et al*, 2004) (Obara *et al*, 2001) (Anderson *et al*, 2006). HA are highly polar molecular that attract water. An increase in HA and aquaporin channels results in increased hydration, this solubilises the collagen, causing dispersal and a disarrangement of the collagen fibres. HA also decreases the interactions between collagen and fibronectin, which causes further disorganisation of the collagen fibres (Timmons *et al*, 2010) (Word *et al*, 2007).

1.7.2- Progesterone Withdrawal

Progesterone obstructs contractions and administering drugs that inhibit progesterone during pregnancy causes a termination of the pregnancy or preterm birth, this evidence suggest that progesterone is essential for maintaining pregnancy. In most mammals there is a reduction in circulating free progesterone before parturition. In non-human primates (great ape and old-world monkeys) and humans, progesterone levels remain elevated throughout pregnancy and birth, thus there is a hypothesis of a "functional" withdrawal of progesterone before parturition (Csapo and Pinto-Dantas, 1965). Functional withdrawal of progesterone is the concept that progesterone sensitivity in human myometrium is lost through several diverse molecular pathways. Through a combination of the loss in sensitivity to progesterone by the myometrium and the upregulation of CAPs, parturition is achieved (Brown *et al*, 2004).

One way a functional withdrawal of progesterone can be achieved is through changes in PR expression in the myometrium, however the evidence for this is differing. When PR expression in myometrial biopsies obtained from women in labour (IL) and those not in labour (NIL) were compared, it showed a decrease in PR expression in biopsies from women IL (How *et al*, 1995). A study by Bernard *et al*, showed no change in myometrial PR expression, when comparing IL and NIL samples (Bernard *et al*, 1988). Conflictingly, another study illustrated that PR expression increased in the myometrium during labour (Rezapour *et al*, 1997).

Changes in progesterone receptor isoform expressed in the myometrium, may be one method for functional progesterone withdrawal. The PR isoform expressed in the myometrium of the pregnant rhesus macaques is dependent on the gestational age; from PRB dominance in mid gestation to PRA dominance during labour (Haluska *et al*, 2002). PRA has been shown to repress PRB activated transcription. Using a progesterone responsive gene in a luciferase reporter assay, it was found that only PRB expression was able to activate transcription of the progesterone responsive gene. Co-expressing of PRA and PRB, inhibited PRB mediated transcriptional activation (Pieber *et al*, 2001). The increase in PRA expression in late gestation leading to an inhibition of PRB mediated quiescent pathways, without the lowering of progesterone, is another example of "functional" progesterone withdrawal.

Increasing metabolism of progesterone into inert metabolites has been proposed as one of the mechanisms of functional progesterone withdrawal. Both 5α-reductase and 20-α-hydroxysteroid dehydrogenase (20α-HSD) metabolise progesterone into the inactive progestin: dihydroprogesterone. One study found that the activity of 20α-HSD increased in the amniochorion when term was reached, subsequently more dihydroprogesterone was detected (Mitchell and Wong, 1993). Another study also found increased expression of 20α-HSD in myometrium biopsies collected from women who were undergoing term and preterm labour, compared to women NIL resulting in unliganded progesterone receptors (Nadeem *et al,* 2016). Furthermore, the creation of knockout mice which lack the 5α-reductase enzyme, resulted in the mice failing to deliver during labour due to impaired cervical ripening, which suggest

that 5α-reductase is important in inhibiting progesterone signalling for successful parturition (Mahendroo *et al,* 1999).

PR has numerous co-factors that aid its ability to activate or repress transcription of genes. Changes in co-factors can inhibit progesterone activity, without a need for a reduction in progesterone concentration i.e. “functional progesterone withdrawal”. One of the co-factors of PR is steroid hormone co-activator (SRC) (Onate *et al*, 1995). SRC has inherent histone acetyltransferase activity (HAT) in its carboxyl terminus and can acetylate histone 3 and 4. Acetylation of histones causes chromatin opening, which facilitates transcription (Spencer *et al*, 1997). Myometrial samples from IL have a reduced expression of SRC2 and SRC3 in compared to those NIL. Additionally, myometrial samples from women IL were found to have decreased acetylation of histone 3 (Condon *et al*, 2003).

Inflammation plays a vital role in cervical ripening, foetal membrane rupturing and enhancing myometrial contractions. Inflammation may also play a role in functional progesterone withdrawal. Prior to birth, pro-inflammatory interleukins are elevated (Young *et al*, 2002). Many of the interleukins activate the transcription factor NF-κB, which in turn binds directly to PR and inhibits it ability to activate transcription (Kalkhoven *et al,* 1996). Amnion cells obtained before and during labour showed that there was an increased activity of NF-κB during labour (Allport *et al*, 2001). Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) is an activator of contractions in the myometrium and a pro-inflammatory effector. $PGF_{2\alpha}$ has been found to upregulate expression of PRA,

but not PRB. Therefore, $PGF_{2\alpha}$ can inhibit the pro-quiescent effects of PRB, by enhancing the PRA induced repression of PRB (Madsen *et al*, 2004).

1.7.3 - Role of Oxytocin in the Stimulation Phase

Many studies have shown that oxytocin plays an extensive role in maintaining uterine contractions, for successful parturition. Oxytocin is synthesised in the large magnocellular neurones of the hypothalamus; whose axons terminate in the posterior pituitary gland. Oxytocin is a short neuropeptide hormone that is conjugated to a carrier protein (neurophysin), it is initially synthesised as an inactive precursor peptide and packed into secretory granules in the Golgi apparatus. The granules transport the precursor protein along the axon and it simultaneously undergoes post-translational modifications, to turn it into a mature active neuropeptide. Depolarisation at the nerve terminals in the pituitary gland causes oxytocin to be exocytosed into the blood stream (Brownstein *et al*, 1980).

There is overwhelming evidence indicating that oxytocin plays a role in parturition, there is more evidence to suggest that oxytocin is involved in the expulsion phase of labour rather than initiation of labour (Blanks and Thornton, 2003). Oxytocin secretion is pulsatile and is peaks with the expulsion of the foetus. In sheep, cow and rhesus monkey, maximal concentration of oxytocin is detected at birth (Glatz *et al*, 1981) (Landgraf *et al*, 1983) (Hirst *et al*, 1993). In the rat, large pulses of oxytocin are secreted when each pup is expelled (Higuchi *et al*, 1986). Therefore, there is

considerable evidence showing that oxytocin is important for the expulsion of the foetus.

Studies in humans have shown widespread differences in measured oxytocin levels before and during labour. The discrepancies can be ascribed to the difficulty in measuring oxytocin levels, as oxytocin is released in short pulses. (Blanks and Thornton, 2003). However, a study by Fuchs *et al* was able to overcome the difficulties in measuring oxytocin release in humans, and they found oxytocin levels in the blood was higher in women undergoing spontaneous labour compared to non-labouring women. Oxytocin was found to be released into the blood stream in discrete pulses that had a short duration. The frequency of the pulses increased during labour (Fuchs *et al*, 1991). Despite the increase in secreted oxytocin during labour, oxytocin knockout mice experience normal deliveries. Although, the offspring from the oxytocin null mice die, but this is because of the inability of the mother to lactate. Therefore, in mice oxytocin is vital for lactation but not for parturition (Nishimori *et al*, 1996) (Young *et al,* 1996). Nonetheless, there is a lot of evidence that oxytocin does play a role in parturition and due to this role, the oxytocin receptor (OTR) is tightly regulated during pregnancy (Blanks *et al*, 2007) In mice and rats the OTR promoter has a full ERE site, the human OTR promoter contains three half palindromic ERE sites (Kubota *et al*, 1996) (Bale and Dorsa, 1997) (Inoue *et al*, 1994). Treatment of ovariectomized rats with oestrogen increases expression of OTR and strengthens binding of oxytocin to OTR (Larcher *et al*, 1995). PR antagonist (RU-486) increases OTR mRNA levels in pregnant rat uterus (Fang *et al*, 1997).

The oxytocin receptor is a GPCR that is coupled to $G_{\alpha q}$. Activation of $G_{\alpha q}$ coupled GPCR stimulates contraction in smooth muscle cells by increasing intracellular calcium concentrations. There is also some evidence that oxytocin increases inward current of L-VOCC, external removal of calcium decreases oxytocin induced rise in intracellular calcium concentrations (Arnaudeau *et al*, 1994). The effects of oxytocin on L-VOCC may explain why oxytocin augments the plateau element of the complex AP in the human myometrium (Nakao *et al*, 1997) (Kawarabayashi *et al*, 1986). Oxytocin is also involved in calcium sensitisation by activation the $G_{12/13}$/rhoA/ROCK pathway (Somlyo and Somlyo, 2000). Inhibition of ROCK reduces the force measured in rat myometrium after treatment with oxytocin (Mckillen *et al*, 1999) (Tahara *et al*, 2002). Oxytocin also causes transcriptional changes by activating the MAPK pathway, and this may lead to an increase in cyclo-oxygenase (COX-2) activity, which subsequently causes an increase in $PGF_{2\alpha}$ production (Figure 1.12) (Kim *et al*, 2015). In summary, the role of oxytocin/OTR in the uterus is not to initiate parturition but to aid parturition during the stimulation phase, by increasing the frequency and force of the contractions (Shmygol *et al,* 2006).

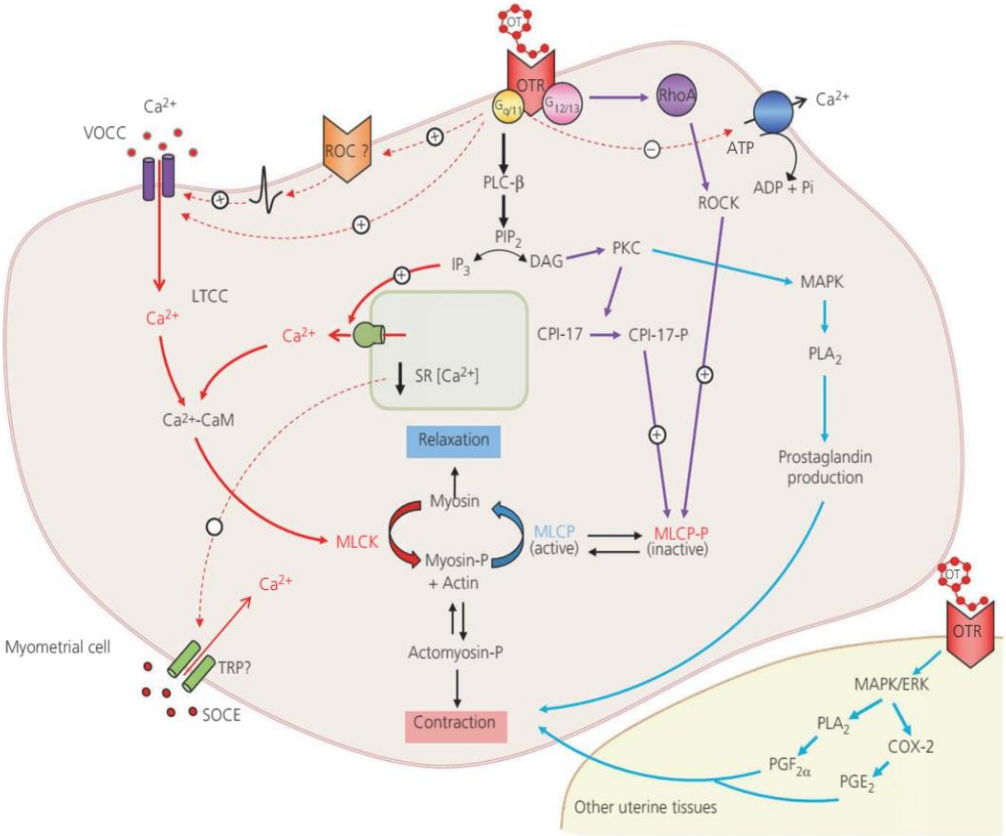


Figure 1.12 - *Oxytocin Mediated Pathways Leading to Contraction in the MSMC.* Red pathways indicate signalling pathways with direct influences on $[Ca]_I$, whereas purple and turquoise lines indicate Ca^{2+}-independent pathways to contraction, including Ca^{2+}sensitisation (purple lines) and the production of prostaglandins (turquoise pathways). Dotted lines indicate where mechanisms are not yet fully determined. Abbreviations: COX-2, cyclooxygenase-2; ROC, Receptor operated channel; VOCC, voltage operated Ca^{2+} channel; LTCC, L-type Ca^{2+} channel; TRP, transient receptor potential channel; Ca^{2+}-CaM, Ca^{2+}-calmodulin complex; ERK, extracellular signal-regulated protein kinase; SOCE, store-operated Ca^{2+} entry. Figure from Arrowsmith and Wray, 2014

1.7.4- Role of Prostaglandins in the Stimulation Phase

Prostaglandins (PG) are significant regulators of parturition, and are involved in membrane rupture, cervical ripening and myometrial contractions. Prostaglandins are synthesised in the amnion, chorion and the decidua from membrane phospholipids. Phospholipase A2 (PLA_2) converts membrane phospholipids into arachidonic acid (AA), which is then converted into prostaglandin H2 (PGH2), by the enzyme COX-2. PGH2 can then be converted into various eicosanoids including prostaglandins by prostaglandin synthases (PGE_2, PGD_2 and $PGF_{2\alpha}$ synthases) (Gibb, 1998). COX-1, PLA_2 and $PGF_{2\alpha}$ receptor null mice all fail to undergo labour due to a failure in luteolysis and progesterone withdrawal, this shows the importance of PG in initiating labour in mice (Uozumi *et al*, 1997) (Sugimoto *et al*, 1997) (Langenbach *et al*, 1995). Women who fail to initiate labour and were induced with oxytocin showed a decrease in expression of PLA_2 in their myometrium compared to women who underwent spontaneous labour, this shows the importance of prostaglandins in the activation of contraction (MacIntyre *et al*, 2009).

Prostaglandin contributes to the pathway to parturition and with increasing gestational duration there is an increase in prostaglandin concentration in the myometrium of rats (Gu *et al*, 1990) and the amnion of Rhesus monkeys (Walsh *et al*, 1984). Additionally, in humans, PG concentration rises in amniotic fluid prior to the onset of labour (Romero *et al*, 1996). The increase in prostaglandin levels is thought to be due to an increase in COX-1 expression (Mijovic *et al*, 1997) and a reduction in 15-hydroxyprostaglandin dehydrogenase (PGDH) activity, during labour

(Giannoulias *et al*, 2002) (Johnson *et al*, 2004). PGDH is an enzyme that metabolises prostaglandins, so a reduction on PGDH leads to an increase in prostaglandins.

Prostaglandins bind to GPCR receptors expressed in MSMCs. The $PGF_{2\alpha}$ and PGE_2 isoforms of prostaglandin bind to the receptors EP1/EP3 and FP, respectively and activation of these receptors causes the human myometrium to contract (Senior *et al*, 1998). The molecular mechanism by which prostaglandins can cause myometrial contraction is similar to the mechanism employed by oxytocin, as prostaglandin receptors are coupled to $G_{\alpha q}$, and activate phospholipase C. Moreover, $PGF_{2\alpha}$ is able to regulate transcription in the myometrium, by upregulating the transcription of pro contractile genes such as: COX-1, OTR and Cx43 (Xu *et al*, 2013).

1.8- Phospholipase C Like 1: Structure, Function and Possible Role in Uterine Quiescence

1.8.1 - PLCL-1: Discovery, Structure and Binding Partners

Phospholipase C like 1 was first discovered as a novel IP_3 binding protein. Kanematsu et al, used an IP_3 affinity column to look for binding partners of IP_3 in rat brain cytosol. Two binding partners of IP_3 were discovered, an 85kDa and a 130kDa protein. Amino acid sequencing revealed that the 85kDa protein was the δ_1-isomer of phospholipase C (PLC-δ_1), and that the 130kDa was an unidentified novel protein that was named p130 (Kanematsu *et al*, 1992).

DNA sequencing of p130 found that it had significant sequence homology with PLC-δ_1. The pleckstrin homology (PH) domain and the catalytic X and Y domain of PLC-δ_1 were found to have 35.2%, 48.2% and 45.8% homologies with the corresponding domains in p130, respectively. P130 was then cloned into COS-1 cells and was again found to bind to IP_3 using an affinity column. As p130 had significant structural similarities with the catalytic domains of PLC-δ_1 and it was able to bind to IP_3, the ability of p130 to hydrolyse PIP2 into DAG and IP_3 was examined. It was found that p130 had no catalytic capabilities. Due to the structural similarities between p130 and PLC-δ_1 and the catalytic inactivity of p130, p130 was renamed phospholipase C-related, but catalytically inactive protein 1 (PRIP-1), it is also known as phospholipase c like 1 (PLCL-1) (Kanematsu *et al*, 1996).

Northern blot analysis of tissues from rat showed that the highest expression of PLCL-1 mRNA to be in the cerebellum and cerebrum followed by the kidney, lung and spleen, in this order. There was significantly less PLCL-1 mRNA observed in the heart, liver or skeletal muscle. Using situ hybridisation, it was shown that in the cerebellum, the PLCL-1 mRNA was found in the granular cell and Purkinje cell layers, and cerebellar nuclei. In the cerebrum, the PLCL-1 mRNA expression was concentrated in hippocampal pyramidal cells, dentate granule cells and pyramidal and/or granule cells of the cerebral cortex (Matsuda *et al*, 1998).

Using protein trypsinisation and SDS/PAGE analysis; the different domains present in PLCL-1 were elucidated. Trypsin treatment of PLCL-1, discovered 4 major polypeptide

bands of the sizes: 55, 33 and 25kDa and a minor band at 48kDa. With increasing trypsinisation time a new band at 40kDa appeared and the three major bands disappeared, apart from the band at 25kDa. The trypsinisation pattern of PLCL-1 was found to be very similar to PLC-δ_1, suggesting that the two proteins have similar domains presents. The major difference between the two proteins, apart from the lack of catalytic activity of PLCL-1, was the unique 25kDa polypeptide present in the C terminus of PLCL-1. The 25kDa polypeptide was found to be resistant to cleavage and rigid in structure, and this domain was termed the D domain (Kanematsu *et al*, 2001) (Figure 1.13).

Figure 1.13 - ***Schematic Representation of the Differences in Structure Between PLCL-1 and PLC-δ and the Domains Present in Each Protein*.** Abbreviations: PH (Pleckstrin domain).

The four amino acids: His311, His356, Asp343 and Glu390 in the X domain of PLC-δ1 are crucial for its catalytic activity as they function as stabiliser amino acids for a calcium–substrate complex (Essen *et al*, 1996). Two of these four amino acids, His356 and Glu390 in PLC-δ1, are not conserved in PLCL-1 and are substituted by asparagine and glycine, respectively, and this may explain the lack of catalytic activity of PLCL-1

(Kanematsu *et al*, 2001). Apart from IP_3, there are 6 other binding partners of PLCL-1 and they include: phosphatase 1c (PP1c), phosphatase 2A (PP2ac), Akt, syntaxin SNAP-25, GABARAP and $GABA_A$ receptor (Figure 1.14).

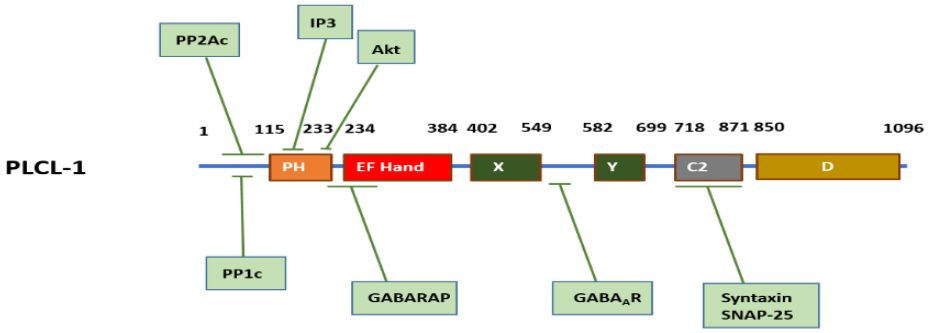


Figure 1.14 - *The Known Binding Partners of PLCL-1.*

1.8.2 - PLCL-1: Possible Role in Reproduction

PLCL-2 is an isomer of PLCL-1, that was discovered to also be able to bind to IP_3 but display no catalytic PLC activity (Otsuki *et al*, 1999). Matsuda et al, created transgenic double knockout mice whereby the genes for PLCL-1 and PLCL-2 were knocked out. When transgenic male and female mice were cross bred together, it was observed that they had reduced number of pups and longer intervals between litter events, compared to wild type mice. This observation was the first to signify that PLCL-1/PLCL-2 might play a role in reproduction. Following on from the initial observation, the reproductive organs of the transgenic mice were examined. The only difference perceived between the organs of wild type and transgenic mice, was found in the

uterus (Figure 1.15). The uteri of the transgenic mice were smaller than wild type, although there was no difference in the wall thickness of the uteri.

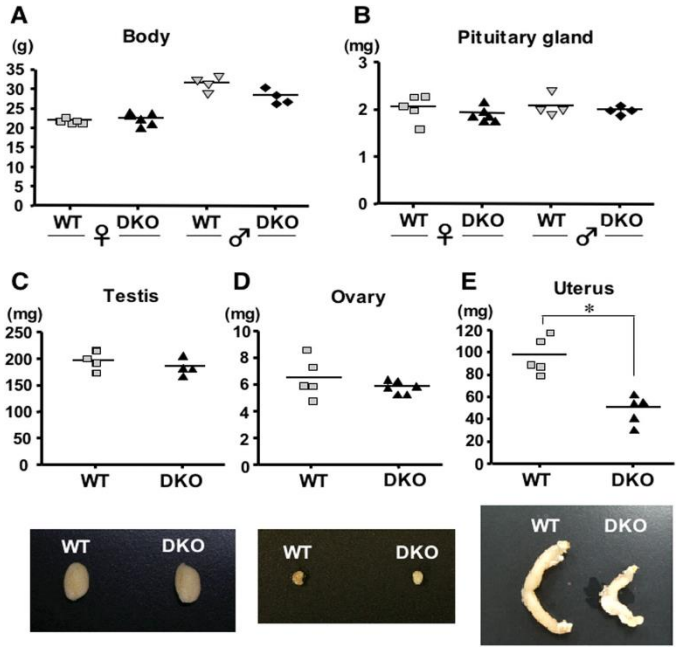


Figure 1.15 - *Wet weights of WT and Knockout Mice Reproductive Organs*. Bodies (A) were weighed, and pituitary gland (B), testis (C), ovary (D), and uterus (E). Figure from Matsuda *et al*, 2009.

To further characterise the reproductive physiology of the transgenic mice; the oestrous cycle was studied, from vaginal smears. Knockout mice had longer oestrus days compared to wild type, and this indicated dysfunction in gonadotropin levels of the knockout mice. The knockout mice had a higher basal level of both LH and FSH in their plasma compared to wild type mice (Figure 1.16). Elevated LH/FSH levels could potentially influence progesterone and oestrogen levels. Serum progesterone was significantly lower in knockout mice compared to wild type, but no change was seen in oestrogen levels.

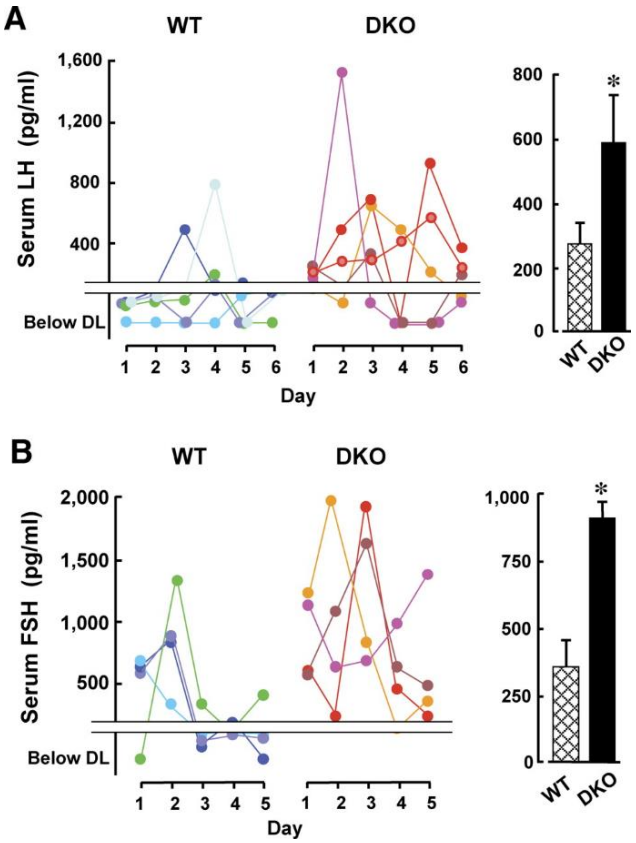


Figure 1.16 - ***Blood Levels of LH and FSH in Wild Type and Knockout Mice.*** **(**A) LH levels, (B) FSH levels of 5 mice. Different colours represent measurements from the different mice. Figure from Matsuda *et al*, 2009.

1.8.3 - PLCL-1: Possible Role in Calcium Signalling and Maintaining Uterine Quiescence

Since PLCL-1 binds to IP_3, and IP_3 releases intracellular calcium from the ER; the possible role PLCL-1 may play in the regulation of calcium concentration was investigated. Increasing concentrations of IP_3 added to COS-1 cells, caused an equivalent surge in intracellular calcium concentrations. However, IP_3 mediated release of calcium was reduced when the PH domain of PLCL-1 was overexpressed. Full length PLCL-1 was also able to decrease release of calcium from intracellular

stores. Bradykinin activates PLC and causes an increase in cytosolic calcium levels. PLCL-1 overexpression dampened bradykinin induced increase in intracellular calcium concentrations; this experiment was done in the absence of any external calcium, suggesting the PLCL-1 effects are on intracellular calcium stores (Takeuchi *et al*, 2000). Knocking out PLCL-1 in mice neuronal cells causes an increase in IP_3 mediated calcium release (Harada *et al*, 2005).

PLCL-1 was found to be a progesterone responsive gene in human endometrial stromal cell (HESC). Treating cells with progesterone analogue: medroxyprogesterone acetate (MPA) in conjunction with cAMP, increased expression of PLCL-1, both at the mRNA and protein level. PLCL-1 knockdown decreased intracellular calcium levels in HESCs (Muter *et al*, 2016).

Myometrial strips were collected from women who either in labour (IL) or not in labour (NIL), thereafter, high-thorough put RNA sequencing was done on extracted RNA. PLCL-1 mRNA was found to be significantly down-regulated in women who were IL compared to those that were NIL (Chan *et al*, 2014) (Sharp *et al*, 2016). The results from the RNA sequencing coupled with the fact that PLCL-1 is a progesterone responsive gene and that PLCL-1 decreases intracellular calcium concentration, signals that PLCL-1 may play role in maintaining uterine quiescence (Muter et al, 2016) (Harada *et al*, 2005). There is also evidence showing a reduction in PLCL-1 expression in the myometrium of labouring mice (Migale *et al,* 2016).

Aims

The hypothesis of this thesis is that PLCL-1 is downregulated in the labouring myometrium because it is a pro-quiescent protein. The overall aim of this thesis is to understand the function of PLCL-1, the mechanism in which it acts as a pro-quiescent protein and can PLCL-1 be used as a future tocolytic target or as a biochemical marker for a preterm diagnostic test. Calcium imaging will be used to see if PLCL-1 has a similar effect on intracellular calcium concentrations in MSMC as it does in COS-1 cells, neuronal cells and HESCs. As changes in calcium have been shown to effect transcription; using RNA-sequencing, the effect of PLCL-1 on the transcriptome of MSMCs can be explored and identify if PLCL-1 affects transcription of pro-quiescent genes and CAPs. One explanation for PLCL-1 being downregulated in the myometrium is that it is an effector protein for progesterone. RT-PCR, western blotting, and ChIP-qPCR will be used to see if PLCL-1 is a progesterone responsive gene. Finally, PLCL-1 might be downregulated not just in the labouring myometrium, but also in the maternal systemic circulation. Collecting blood samples from women IL and NIL will show if there is a universal downregulation of PLCL-1 during labour, this will highlight if PLCL-1 is a potential biochemical marker for a reliable preterm diagnostic test.

Chapter 2

Materials and Methods

2.1 - Antibodies

Table 2.1 - Antibodies

Antibody	Dilution	Manufacturer
PLCL-1	1:1000	Sigma
β-Actin	1:5000	Sigma
Desmin	1:100	Abcam
Vimentin	1:100	Dako
CACNA1C	1:100	Abcam
Anti-Goat-Alexa Fluor 488	1:100	Thermofisher
Anti-mouse Alexa Fluor 488	1:200	Thermofisher
Anti rabbit Alexa Fluor 488	1:200	Thermofisher
PDE7B	1:1000	Abcam
Anti-rabbit HRP	1:5000	Dako
Anti-mouse HRP	1:5000	Dako
Anti-goat HRP	1:5000	Dako
CTCF	2µg/per sample	Merck Millipore
IgG	2µg/per sample	Merck Millipore
ZNF143	2µg/per sample	Abcam
YY1	2µg/per sample	Abcam

2.2 – siRNAs and Vectors

Table 2.2- siRNA and Vectors

Description	Manufacturer
siControl: ON-TARGET plus Non-targeting Pool 1.	Dharmacon Horizon
siPLCL-1: On Target plus Human PLCL-1 siRNA SMARTPOOL	Dharmacon Horizon
MGC Human PLCL-1 Full sequence cDNA clone in gateway backbone vector pCMV-SPORT6	Thermo- Fisher

2.3 – Primers

Table 2.3 - Primers

Gene	Forward	Reverse
PLCL-1	GCAGCAGCATCATCAAGG	GCTGCTGAAAGACACGGTT
L19	GCGGAAGGGTACAGCCAAT	GCAGCCGGCGCAAA
OTR	GGGGAGTCAACTTTAGGTTCG	GAATCCTCTACCGGCCACAA
FP	GGAGCCCATTTCTGGTTACAA	GATTCCATGTTGCCATTCGGA
Desmin	TTCTTCCATCCCAGGACACC	GATGGGCTATGTCGCTGTTG
Vimentin	AAGGAGGAAATGGCTCGTCA	AGCTTCCTGTAGGTGGCAAT
CACNA1C	GAAGATGACTGCTTATGGGG	AGCAGGTCCAGGATGTTGAA
PDE7B	TCCTGGATGAAGTTGCTGGAT	AATGCCGTGAAATCTCTGAGC
PDE2A	AGGACGCCTTGCTGAGTCT	TAGACAGTTTCCACTCGGGG
PDE3A	GGCCAAGAGGATCACAGTCTT	TGGAAGAGAAGGGTCTGCC
WNT5A	CAGACGTTTCGGCTACAGAC	CCCAGTTCATTCACACCACA
PAQR5	ACCTACTGGCCTTGCCAATC	TTGCTCATGGAACACCTGGG
CACNA1H	CACTTTCCCCAGCCCAGA	CACAGCGAGTGAATGGAGC
WNT7B	CACAGAAACTTTCGCAAGTGGA	ATGACAGTGCTCCGAGCTTCA
EFHD1	CCGTACTGTCCCTGTGAAGG	CCAGCGTCATACTGCAACTC
ChIP-qPCR	ACCAGAGGTCACTGCTACTCT	TGGGCTACAACCAGCCAGA

2.4 – Kits

Table 2.4 - Kits

Kit	Manufacturer
QuantiTect Reverse Transcription Kit	Qiagen, Germany
QIAquick Gel Extraction Kit	Qiagen, Germany
QIAquick PCR Purification Kit	Qiagen, Germany
PAXgene Blood RNA Kit IVD	Qiagen, Germany
QIAamp ccfDNA/RNA Kit	Qiagen, Germany
cAMP assay kits	Cisbio, France
Basic Nucleofector Kit for Primary Mammalian Smooth Muscle Cells	Lonza, Germany
Human Phospho-MAPK Array Kit	R&D Systems
GeneJet Endo-free Plasmid maxiprep kit	Thermo Scientific, Lithuania

2.5 – Chemical Reagents

Table 2.5 – Chemical Reagents

Product	Manufacturer
2-[(2-Hydroxy-1,1-bis(hydroxymethyl)ethyl)amino]ethanesulfonic acid (TES)	Sigma Aldrich, USA
30% Acrylamide/Bis Solution	Bio-Rad, UK
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (HEPES)	Sigma Aldrich, USA
Ammonium Persulphate (APS)	Thermofisher, USA
Boric Acid	Thermofisher, USA
Calcium Chloride ($CaCl_2$)	Sigma Aldrich, USA
Chloroform	Sigma Aldrich, USA
Dimethyl sulfoxide (DMSO)	Life Technologies, USA
Ethidium Bromide	Sigma Aldrich, USA
Ethylenediaminetetraacetic acid (EDTA)	Sigma Aldrich, USA
Glycine	Sigma Aldrich, USA
Isopropanol	Sigma Aldrich, USA
Lithium Cholride (LiCl)	Sigma Aldrich, USA
Magnesium Chloride ($MgCl_2$)	Sigma Aldrich, USA
Magnesium Sulphate ($MgSO_4$)	Sigma Aldrich, USA
N,N,N',N'-Tetramethylethylenediamine (TEMED)	Sigma Aldrich, USA
NP-40	Thermofisher, USA
Pluronic Acid	Sigma Aldrich, USA
Potassium Chloride (KCl)	Sigma Aldrich, USA

Potassium phosphate monobasic (KH_2PO_4)	Sigma Aldrich, USA
Sodium Bicarbonate ($NaHCO_3$)	Sigma Aldrich, USA
Sodium butyrate	Sigma Aldrich, USA
Sodium Chloride (NaCl)	Sigma Aldrich, USA
Sodium Deoxycholate	Sigma Aldrich, USA
Sodium dodecyl sulfate (SDS)	Sigma Aldrich, USA
STAT-60	Amsbio, UK
Tris	Sigma Aldrich, USA
Triton X-100	Sigma Aldrich, USA
Tween 20	Sigma Aldrich, USA

2.6 – Miscellaneous Materials

Table 2.6 – Miscellaneous Materials

Product	**Manufacturer**
Halt protease cocktail inhibitor	Thermofisher, USA
Phosphatase Inhibitor Cocktail 2	Sigma Aldrich, USA
Phosphatase Inhibitor Cocktail 3	Sigma Aldrich, USA
Collagenase Type IV	Life Technologies, USA
DMEM (High Glucose/Glutamax)	Life Technologies, Paisley Scotland
Penicillin/Streptomycin	Thermofisher, USA
Foetal Bovine Serum	Hyclone, South America
Trypsin/EDTA (0.25%)	Life Technologies, Paisley Scotland

Lonza's Basic Nucleofector Solution for Mammalian smooth muscle cells	Lonza, Germany
Ampicillin	Sigma Aldrich, China
LB powder	Sigma Aldrich, USA
Agarose Powder	Life Technologies, USA
Sybr-green Master Mix	Thermofisher, USA
Loading dye	Thermofisher, Lithuania
Bovine Serum Albumins (BSA)	Sigma Aldrich, USA
Bradford Reagent	Sigma Aldrich, USA
Ripa Lysis Buffer	Millipore, USA
mini protease inhibitor tablet	Roche, Germany
phosphatase inhibitor cocktail	Sigma Aldrich, USA
Nupage Sample Buffer	Life Technologies, USA
DTT	Sigma Aldrich, Switzerland
Nitrocellulose Membrane	Amersham, Germany
Milk Block Powder	Bio-Rad, UK
ECL Detection Reagent	Amersham, Germany
25 mm coverslips	VWR, Germany
Indo-1-AM	Life Technologies, USA
Fluo4-am	Life Technologies, USA
Goat Serum	Sigma Aldrich, USA
VECTASHIELD® Antifade Mounting Medium with DAPI	Vector, USA
Glass Slides	VWR,USA

Nuclease Free Water	Life Technologies, Netherlands
Bioruptor Picotubes	Diagnode
Lobind Tube	Eppendorf, Germany
Protein A/G Beads	Thermofisher, USA
DynaMag	Thermofisher
Proteinase K	Life Technologies, Netherlands
PAXgene Blood RNA Tubes	BD Bioscience, UK
BD Vacutainer Venous Blood Collection Tubes	BD Bioscience, UK

2.7 – Criteria for Collecting Myometrial Biopsies

Myometrial biopsies for women NIL were taken from an incision of the midline from elective caesareans from full term women (≥37 weeks) before the onset of labour. The medical indications for the elective caesareans were due to: placenta previa, malpresentations and more than one previous history of a caesarean. There were also biopsies taken from elective caesareans for non-medical reasons due to maternal wish. Emergency caesareans due to a failure to progress were excluded. Any patient that was given oxytocin was excluded.

Myometrial biopsies from labouring women were undergoing caesareans for reasons of undiagnosed breech or large baby/cephalopelvic disproportion. Labour was defined as regular contractions (<3 min apart), membrane rupture, and cervical dilatation (>2 cm) with no augmentation.

2.8 - Cell Culturing

Myometrial biopsies from an incision of the midline at caesarean section undertaken for clinical reasons. Biopsies were stored in 15ml Krebs buffer (133mM NaCl, 4.7mM KCL, 11.1mM, 1.2mM $MgSO_4$, 1.2mM KH_2PO_4, 10mM TES and 2.5mM $CaCl_2$). 40mg of collagenase type IV was added to 20ml of DMEM, which contained 1% penicillin/streptomycin. The blood vessels were removed, and the biopsy was cut up using a scalpel for 15 minutes. The biopsy was then placed in 20ml DMEM media and incubated in a shaking water bath for 3 hours at 37^{o}C. The sample was then centrifuged at 215 x g for 3 minutes. The supernatant was removed, and the pellet

was resuspended in 20ml PBS buffer, the sample was centrifuged at 215 x g for 3 minutes. The supernatant was removed, and the pellet was resuspended in 20ml PBS buffer, the sample was then centrifuged at 215 x g for 3 minutes. The sample was then resuspended in 25ml standard DMEM, which contained 1% penicillin/streptomycin and 10% foetal bovine serum. The primary myometrium cells were grown in 37°C and 5% CO_2, the cells were not used after passage 4.

2.9 - Cell Transfection-Electroporation

Primary MSMCs were grown until 70% confluency. Media was removed from primary myometrium cells and washed twice with 15ml PBS buffer. The PBS buffer was discarded and 5ml of 0.25% trypsin/EDTA was added to the cells. The cells were incubated for 5 minutes in 37°C and 5% CO_2. 10ml of DMEM media was added to detached cells and centrifuged at 215 x g for 5 minutes. The supernatant was removed, and the pellet was resuspended in 15ml of DMEM media, the number of cells/ml were counted using Luna automated cell counter. For each transfection 1 $x10^6$ cells per ml were used, the cells were then spun at 215 x g for 5 minutes. The supernatant was removed, and the cells were resuspended in 100µl of Lonza's Basic Nucleofector Solution for Mammalian smooth muscle cells. 200nM of siControl or siPLCL1 or 1µg of PLCL1 plasmid DNA was added the cells. The mixture was then placed in an Amaxa cuvette and then inserted into the Amaxa machine (Nucleofector 2b Device), an electric field was applied to the cells using the A-023 Amaxa programme. 1ml of DMEM media was added to the cuvette. The transfected cells

were then split into 4 wells of a 6 well plate, 2ml of DMEM media was added to each well. Cells were grown for 48 hours post-transfection and then harvested.

2.10 - Plasmid Extraction from Transformed Cells

Glycerol stock of transformed *E.coli* with PLCL-1 plasmid DNA was thawed at room temperature. 100µg/ml of ampicillin was added to 250ml of LB broth. 1ml of transformed *E.coli* was added to 250ml of LB broth. *E.coli* cells were then grown overnight at 37°C in a shaking rotor. To extract the PLCL-1 DNA plasmid from the cells the GeneJet Endo-free Plasmid maxiprep kit's manual procedure was followed. The cells were centrifuged for 10 minutes at 4000 x g and the pellet was resuspended in 7ml resuspension solution containing 40µl of RNAse solution. 7ml of lysis solution was added to the mixture and it was mixed gently until the solution became clear. The mixture was then incubated at room temperature for 3 minutes. 7ml of neutralisation solution was added to mixture and then mixed gently. 1ml of endotoxin solution was added and mixed gently and thoroughly, the mixture was incubated at room temperature for 10 minutes. 4ml of isopropanol was added to the mixture and mixed gently. The mixture was centrifuged at 4000 x g for 20 minutes. The supernatant was transferred to GeneJet Maxi Endo-free Filtration Column. The column was centrifuged at 500 x g for 2 minutes. The column was discarded and the 8ml of isopropanol was added to the flow through and mixed gently. The mixture was then transferred to another filtration column and was centrifuged at 3000 x g for 3 minutes, this was then repeated to process any remaining lysate through the filtration column. The flow through was discarded and the column was placed back

into the same collection tube. 25ml of isopropanol was added to 75ml of wash solution I, 8 ml of wash solution was then added to the filtration column. The column was then centrifuged at 3000 x g for 2 minutes, the flow through was discarded and the column placed back into the same collection tube. 125ml of ethanol (100%) was added to 75ml of wash solution II, 16ml of wash solution II was added to the filtration column and centrifuged at 3000 x g for 2 minutes. The flow through was discarded the column was placed back into the same collection tube. The column was centrifuged at 3000 x g for 5 minutes, the collection tube was discarded and the column was transferred to a new collection tube. 1ml of elution buffer was added to the centre of the column and was incubated at room temperature for 2 minutes. The column was centrifuged at 3000 x g for 5 minutes to elute the plasmid DNA. The column was discarded and the flow through containing the purified plasmid DNA was stored at -20^{o}C.

2.11 - 1% Agarose Gel Preparation

1g of agarose powder was added to 100ml of 1X TBE buffer (0.1M TRIS, 0.1M Boric Acid, 2.5mM EDTA, pH 8.0). The mixture was heated up until all the agarose powder solubilised. The mixture was incubated at room temperature for 30 minutes and thereafter 2µl of ethidium bromide was added. The mixture was placed in a gel holder with a well comb to solidify into a gel.

2.12 - Primer Optimisation

Primers were chosen using Primer-Blast NCBI. 19µl of Sybr green master mix containing 20µM of reverse and forward primers were added to each well, in a 96 well plate. 1µl of cDNA or nuclease free water was added to each well. The above steps were repeated twice more, creating triplicate repeats for each primer set. The triplicate samples were pooled together and loading dye was added. The samples were run on 1% agarose gel for 45 minutes at 100V. The DNA was visualised on the gel using UV light. The DNA was removed from the gel using Qiagen gel extraction kit. The purified DNA was then in succession diluted from 100pg/µl to 10ag/µl with a 10-fold dilution factor in nuclease free water. The diluted samples were amplified using qPCR. The log of the concentrations were plotted against the CT values and the primer efficiency was calculated using the equation: Primer efficiency = 10 (-1/gradient of the line).

2.13 - Qiagen Gel Extraction Kit

DNA was removed from the gel using a scalpel. The gel was weighed and 3x volumes/per 1x volume of buffer QG was added. The gel was incubated in a heat block set at 55°C for 10 minutes. 1x gel volume of isopropanol was added and the mixture was placed in column and centrifuged at 16000 x g for 1 minute. The flow through was discarded and 750µl of buffer PE was added to the column. The column was centrifuged at 16000 x g for 1 minute and the flow through was discarded. The column was placed in a nuclease free 1.5ml microcentrifuge tube and 50µl of buffer

EB was added to the centre of the column. To elute the purified DNA , the column was centrifuged at 16000 x g for 1 minute.

2.14 - RNA Extraction

The culture media was removed and the cells washed twice with PBS. 200µl of stat-60 was added to each well in a 6 well plate. The cells were scraped thoroughly into 1.5ml nuclease free Eppendorf tube. The samples were kept on ice throughout the RNA extraction procedure and all centrifugation was done at 4^{O}C. 20% of the total volume of ice-cold chloroform was added to the each sample and vortexed vigorously. The samples were then centrifuged at 16000 x g for 30 minutes. The top aqueous phase was then transferred to a new nuclease free Eppendorf tube and 100µl of ice-cold isopropanol was added to each sample. The samples were vortexed thoroughly and stored at -80^{O}C for 30 minutes. The samples were thawed on ice and centrifuged at 16000 x g for 15 minutes. The supernatant was discarded and the pellet resuspended in 500µl of ice-cold 75% ethanol. The samples were centrifuged at 16000 x g for 15 minutes. The supernatant was discarded and the pellet resuspended in 500µl of ice-cold 75% ethanol. The samples were centrifuged at 16000 x g for 15 minutes. The supernatant was discarded and the pellet was air dried for 7-10 minutes, the pellet containing the RNA was resuspended in 30µl of nuclease free water. The total RNA concentration was verified by pipetting 1.5µl of sample on the NanoDrop spectrophotometer. The total RNA samples were stored at -80^{O}C.

2.15 - cDNA Synthesis for RT-PCR

Samples containing extracted total RNA were thawed on ice. The QuantiTect Reverse Transcription Kit manual procedure was followed to make cDNA from total RNA. For each sample: 1µg of total RNA was added to 2µl of gDNA wipeout buffer, and the total volume was made up to 14µl, using RNAse free water. The gDNA wipeout buffer removed any genomic DNA from the sample. The samples were mixed gently and incubated at 42°C for 2 minutes and then placed back on ice immediately. A master mix was created containing 4µl of Quantiscript RT buffer, 1µl RT primer mix and 1µl of Quantiscript Reverse transcriptase for each sample. 6µl of the master mix was added to each sample, thus making the total volume of each sample 20µl. The samples were incubated at 42°C for 30 minutes and thereafter incubated at 95°C for 3 minutes to inactivate the Reverse Transcriptase. The samples were then either stored at -20°C or used for RT-PCR immediately.

2.16 - RT-PCR

19µl RT-PCR master mix 20µl (10 µl Sybrgreen, 0.3 µl forward primer, 0.3 µl reverse primer, 8.4 µl nuclease free water) was added to each well of a 96 well PCR plate. The cDNA samples were thawed on ice and diluted by adding 30µl of nuclease free water. 1µl of diluted cDNA was added to each well of the PCR plate making the total volume in each well 20µl and the total cDNA in each well 20ng. The plate was sealed and then centrifuged at 160 x g for 3 minutes and thereafter placed in the PCR machine. The thermocycling conditions were: i) 50°C , 2 minutes ii) 95°C , 10

minutes iii) 95°C, 15 seconds iv) 60°C ,1 minute for 40 cycles. The PCR results were analysed using the delta delta CT method, with each sample being adjusted to the corresponding CT values from the housekeeper gene (L19). The test samples were normalised to the control samples to calculate the expression fold change between control samples and test samples.

2.17 - HiSeq Sequencing and Sample Preparation

Primary myometrium cells at passages 1-3 were transfected with either siControl or siPLCL-1 (Section 2.9) for 48 hours and then harvested. The RNA was then extracted (Section 2.14). The RNA was then quantified using a Nanodrop spectrophotometer, 1µg/µl of the RNA was then converted into cDNA (Section 2.15) and used in RT-PCR and the rest stored in -80°C. 1.5µl of the total RNA was taken for purity analyses using a 2100 Bioanalyzer (Agilent Technologies). 1µg of total RNA for each sample was then processed for TruSeq Stranded mRNA library preparation.

2.18 - Measuring Protein Concentration

A working solution of BSA was made by diluting the stock solution (10mg/ml) 100-fold in distilled water. 8 standards were made by diluting the working solution of BSA from 10µg/ml to 0µg/ml to a total volume of 500µl. The protein lysates were diluted 20-fold to a total volume of 500µl using distilled water. 500µl of Bradford reagent was added to each test sample and the standards. The mixtures were incubated at

room temperature for 15 minutes. 200µl of the mixture was loaded in triplicate into a well in a 96 well plate end the absorbance was read at 595nm using PHERAstar Fs plate reader. The mean OD from each sample was calculated and the mean OD of the standards were plotted against the concentration for a standard curve. A linear trendline was plotted from the standard curve. The unknown protein concentrations were calculated from the linear line using the equation y=mx + c. The final concentration was calculated using the equation: concentration x dilution factor.

2.19 - Western Blot Analysis

Primary myometrium cells were grown in a 6 well plate at 37^{o}C and 5% CO_2 and transfected with either DNA or siRNA for 48 hours. The cells were washed with PBS twice. 1X Ripa lysis buffer was made by diluting 10x Ripa lysis buffer 10-fold in deionised water and adding one complete mini protease inhibitor tablet and 100µl of phosphatase inhibitor cocktail. The cells were lysed using 200µl of 1x Ripa lysis for each well in a 6 well plate. The lysates were stored at -20^{o}C. 50µg/60µl of total protein was added to 25µl of Nupage sample buffer and 10µl of DTT. The samples were then incubated in 95^{o}C for 5 minutes. A 10% gel (3.25ml 30% acrylamide/bis solution, 2.5ml 1.5M TRIS-HCL pH 8.8, 4ml H_2O, 100µl 10% SDS, 125µl 10% APS, 5µl TEMED) was made in a 0.75mm spacer; isopropanol was added before gel polymerisation. After polymerisation, the isopropanol was discarded and stacking gel (1ml 30% acrylamide protogel,1.5ml 0.5M TRIS-HCL pH 6.8,3.4ml H_2O, 60µl 10% SDS, 80µl 10% APS,5µl TEMED) was added, and then a comb placed before the stacking

gel polymerised. 1X running buffer was made by mixing 100ml of 10x running buffer (1litre; 250mM Tris Base, 1.9 M Glycine, 1 % SDS) with 900ml of H_2O. 30µl of each sample was then run down the 10% gel for ~1¼ hour at 150 volts in ~1 litre of 1X running buffer. One litre of 10X transfer buffer (250mM Tris, 1.9M Glycine) was made. 1X transfer buffer was made by adding 100ml of 10X transfer buffer to 200ml of methanol and 700ml of H_2O. The gel was transferred on to a nitrocellulose membrane at ~10 volts for 2¾ hours in ~1 litre of ice-cold transfer buffer. The gel was discarded, and the membrane was blocked for one hour in 25ml of blocking buffer (5% milk/TBST). The membrane was then incubated in 5ml of diluted primary antibody overnight at 4^oC. The membrane was washed 3 times for 15 minutes using TBST. The membrane was then incubated in 10ml of diluted secondary antibody for 1 hour. The membrane was washed 3 times for 15 minutes each using TBST. The membrane was then incubated in ECL detection reagent for 3 minutes. The membrane was developed using a Syngene chemiluminescence detection machine.

2.20 - Calcium Signalling Assay

Primary myometrium cells were grown on 25 mm coverslips in a 6 well plate until confluent. 1mM of indo-1-AM or 1mM of Fluo4-am stock solution was prepared in DMSO. One litre of 1X PSS buffer was prepared (136mM NaCl, 5mM KCl, 1.8mM $CaCl_2.2H_2O$, 0.25mM $MgCl_2$,10mM Glucose, 5mM HEPES). 30mg of Pluronic acid was dissolved in 120µl of DMSO. For each well in a 6 well plate; 10µl of indo-1-AM or Fluo4-am stock solution and 10µl of Pluronic acid was added to 2ml of PSS buffer and the mixture was vortexed. Each well was washed with 3ml of PSS buffer and the

coverslip transferred to a 35mm dish. The cells were washed twice with 3ml PSS buffer and then incubated with indo1-AM, Fluo4-AM/Pluronic acid mixture for 45 minutes at room temperature. The cells were washed twice with PSS buffer and then left in PSS buffer for 15 minutes at room temperature. The cells were then treated with Oxytocin and the fluorescence change monitored using a fluorescent microscope. For Indo1-am measurements, a perfusion station was connected to an inverted Olympus fluorescent microscope with a water immersion lens (10× objective N.A.0.4). The excitation wavelength was 340nm and the emitted wavelengths at 400 and 475 nm were recorded at 20 frames per second. The microscope was connected to a computer workstation running an Andor software. The ratio between the two wavelengths (F_{400}/F_{475}) was calculated. For Fluo-4am measurements a perfusion station was connected to a Zeiss LSM 510 META confocal microscope with a x 40 oil immersion objective lens**.** Cells were excited at 488 nm using a krypton/Argon laser and emitted light wavelength was recorded at 510 nm at 1 frame per second. The microscope was connected to a computer workstation running an LSM image analysis software. Mean fluorescence intensity was quantified in regions of interest positioned over different cells and stated as a fold increase from time 0 (F/F_0). Calcium traces were plotted, and the baseline was determined, from the baseline the area under curve was calculated using GraphPad PRISM software.

2.21 - Immuno-cytochemistry

Cells were grown on 25 mm coverslips to confluency in a 6 well plate and washed with twice with PBS (2.5ml/per well). The PBS was discarded, and the cells were fixed

using 2.5ml/per well of ice cold 100% methanol at -20°C for 10 minutes. The cells were blocked with 10% goat serum/PBS (v:v) for 30 minutes. Cells were washed with 1% goat serum/PBS (v:v). Primary antibody was diluted in a solution of 1% goat serum/PBS. Cells were incubated overnight at 4°c with 1.5ml/per well of primary antibody solution. The cells were washed using 2.5ml/per well of 1% goat serum/PBS. Secondary antibody was diluted in a solution of 1% goat serum/PBS. The cells were incubated with 1.5ml/per well of the secondary antibody for 1 hour at room temperature, away from any light. The cells were washed using 2.5ml/per well of PBS twice and the PBS was discarded. The excess PBS was gently removed using tissue paper before mounting with one drop/per coverslip of VECTASHIELD® Antifade Mounting Medium with DAPI. Excess mounting medium was removed gently from coverslip edges using tissue paper. Coverslip was placed cell side down on glass slides and adhered onto slides using nail polish on the coverslip edges. Fluorescence was detected using EVOS m5000 fluorescent microscope.

2.22 - Chromatin Immunoprecipitation (ChIP)

Buffer Preparation :

50ml of 10X swelling buffer in nuclease free water: 0.25M HEPES, 0.015M $MgCl_2$, 0.02M KCl, 500µl NP40, pH 7.9.

1X swelling buffer: 10X swelling buffer was diluted 10-fold in nuclease free water, 165µl of protease inhibitor cocktail was added, 1 tablet each of phosphatase inhibitor cocktail 2 and 3 were added . 330µl of 1M sodium butyrate was added.

50ml of 10X SDS lysis buffer in nuclease free water: 0.65M TRIS-HCl, 0.1M EDTA, 0.07M SDS, 0.12M sodium deoxycholate, 5ml Triton X-100, pH 8.1.

1X SDS lysis buffer: 10X SDS lysis buffer was diluted 10-fold in nuclease free water, 165µl of protease inhibitor cocktail was added, 1 tablet each of phosphatase inhibitor cocktail 2 and 3 were added . 330µl of 1M sodium butyrate was added.

1X low salt wash buffer: 0.01% SDS, 1% Triton X-100, 2mM EDTA, 20mM TRIS-HCl, 150mM NaCl, pH 8.0.

1X High Salt buffer: 0.01% SDS, 1% Triton X-100, 2mM EDTA, 20mM TRIS-HCl, 500mM NaCl, pH 8.0.

1X lithium chloride wash buffer: 0.25M LiCl, 1% NP-40, 0.02M Sodium Deoxycholate, 1mM EDTA, 10mM TRIS-HCl, pH 8.0

TE buffer: 10mM Tris, 1mM EDTA, pH 8.0

1X elution buffer: 35mM SDS, 100mM $NaHCO_3$.

1X IP buffer: 0.01% SDS, 1.1% Triton X-100, 1.2mM EDTA, 16.7mM TRIS-HCl, 16.7mM NaCl.

Cells were grown to 80% confluency in 10mm dish and washed with PBS twice before being incubated in serum free DMEM media containing 1% penicillin/streptomycin for 24 hours. The cells were then treated with 1µM MPA, and/or 0.5mM 8-bromo-cAMP (cAMP) for 96 hours at 37°C and 5% CO_2. Cells were washed twice with 10ml/per well PBS. The cells were then fixed with 2ml/per well of 1% formaldehyde/nuclease free water for 10 minutes at 37°C. 200µl of 1.25M glycine was added to each well and incubated at room temperature for 5 minutes. The dish was placed on ice and the cells washed with sterile PBS twice. 1ml of 1X swelling buffer was added to each dish and the dish was placed on ice for 10 minutes. The cells were scraped into 1.5ml Bioruptor Picotubes. The cells were then homogenized for 1 minute. The cells were centrifuged at 16000 x g for 3 minutes at 4°C. The supernatant was discarded, and the samples were resuspended in 500µl of 1X SDS lysis buffer. The samples were sonicated in Diagenode Bioruptor 300, the sonicator was run for 30 cycles with 30 seconds on and 30 seconds off at a high setting at 4°C. The samples were centrifuged at 16000 x g for 10 minutes at 4°C. The supernatant was transferred to a clean 2ml lobind tube. The sonicated chromatin was diluted 10-fold in 1X IP buffer. 20µl/per sample of protein A/G beads was added to a clean lobind tube. The beads were placed in a DynaMag and the liquid was discarded. The tubes were removed from the DynaMag and washed with 20µl/per sample of ice-cold IP

buffer. 1ml of diluted chromatin was added to 20µl of beads. The samples were rotated at 4°C for 3 hours. The samples were placed in the DynaMag and the chromatin supernatant was transferred to a clean lobind tube, 50µl was taken as input control and stored at -80°C. 100µl of protein A/G beads were added to a clean lobind tube (2x tubes), the tubes were placed in the DynaMag, and washed with 100µl of IP buffer per tube. 1ml of ice-cold IP buffer was added to each tube. 2µg of antibody per sample was added to the tubes and the tubes were rotated for 3 hours at 4°C. The antibody/beads tubes were aliquoted into a clean lobind tube (one tube for each chromatin sample) and were placed in a DynaMag and the supernatants removed. 1ml chromatin samples were equally divided; half was added to target antibody/beads and half was added to IgG control/beads. The tubes were rotated at 4°C overnight. The tubes were placed in the DynaMag and the supernatant was discarded. The chromatin/antibody/beads samples were washed with 300µl low salt wash buffer by rotating for 5 minutes at 4C. The samples were placed in the DynaMag and the low salt wash buffer was removed. The washing step were repeated using high salt buffer, then lithium chloride buffer and then the TE buffer. The TE buffer was removed and discarded and each sample was incubated in 250µl of elution buffer for 15 minutes at room temperature. The tubes were placed in the DynaMag, and the supernatant was placed in a clean lobind tube. The input control samples were defrosted and 200µl of elution buffer was added. 50µl of 1M NaCl was added to each sample. The samples were incubated overnight at 65°C. 10mM EDTA, 40mM TRIS-HCl pH 8.0 and Proteinase K (40µg/ml) was added to each sample and the samples were incubated at 55 for one hour. The DNA was purified using QIAquick PCR purification kit and the DNA was used for qPCR.

2.23 - DNA Purification Using PCR Purification Kit

1.5ml of buffer PB was added to 300µl of DNA obtained from ChIP . The mixture was placed in a column and centrifuged at 16000 x g for 1 minute, the flow through was discarded. 750µl of buffer PE was added to the column and centrifuged at 16000 x g for 1 minute, and the flow through was discarded. The empty column was centrifuged at 16000 x g for 1 minute, and the flow through was discarded. The column was placed in nuclease free microcentrifuge tube and 30µl of buffer EB was added to the centre of the column and it was centrifuged at 16000 x g for 1 minute. The purified DNA was stored at -20°C for qPCR.

2.24 - Patient Criteria for Extracting Blood

Whole blood samples were taken from two different groups of women. The first group of women were pregnant between 16-22 weeks and were attending a preterm clinic. These women were deemed at risk for premature labour. The second group of women were women in active spontaneous labour. Any women who had their labour induced by being given oxytocin were excluded. The women who donated their blood were not the same women who donated their myometrial biopsies.

2.25 - Extracting RNA from Whole Blood

Whole blood was collected from women using PAXgene Blood RNA Tubes and incubated at room temperature for 2 hours**.** The PAXgene Blood RNA Kit was used to

extract total RNA. The tubes were centrifuged at 3000 x g for 10 minutes and the supernatant was discarded. The pellet was resuspended in 4ml of RNAse free water and vortexed. The tubes were centrifuged at 3000 x g for 10 minutes, the supernatant was discarded. The pellet was resuspended with 350µl of buffer BR1, and vortexed. The sample was transferred into 1.5ml RNAse free microcentrifuge tube, 300µl of buffer BR2 and 40µl of proteinase K was added to the sample. The mixture was transferred to a column, and then centrifuged at 16000 x g for 2 minutes. The flow through was transferred to a clean, RNAse free 1.5ml microcentrifuge tube and 350µl of pure ethanol was added. The mixture was vortexed and then transferred to a column and centrifuged at 16000 x g for 1 minute. The flow through was discarded and 350µl of buffer BR3 was added to the column, the column was then centrifuged at 16000 x g for 1 minute. The flow through was discarded. 10µl of DNAse 1 and 70µl of buffer RDD was added to the column, and the column was incubated at room temperature for 15 minutes. 350µl of buffer BR3 was added to the column and thereafter centrifuged at 16000 x g for 1 minute, the flow through was removed. 500µl of buffer BR4 was added to the column and centrifuged at 16000 x g for 1 minute and the flow through was removed. 500µl of buffer BR4 was added to the column and centrifuged at 16000 x g for 3 minutes and the flow through was removed. The column was placed in a RNAse free 1.5ml microcentrifuge tube and 40µl of buffer BR5 was added to the centre of the column. The column was centrifuged at 16000 x g for 1 minute. The concentration of the purified RNA was measured using a nanodrop and stored at -80°C for future use. The purified total RNA was used in RT-PCR.

2.26 - Extracting Circulating Cell Free RNA (ccfRNA) from Plasma

Whole blood was collected in BD Vacutainer Venous Blood Collection Tubes and ccfRNA was extracted from plasma using QIAamp ccfRNA kit. The tubes were centrifuged at 2000 x g for 10 minutes at 4°C. The plasma was transferred to a conical tube and centrifuged at 3000 x g for 10 minutes. The supernatant was transferred to a new tube. 300µl of buffer RPL was added for every 1ml of plasma and the mixture was vortexed. 100µl of buffer RRP was added for every 1ml of plasma and the mixture was vortexed and thereafter incubated on ice for 3 minutes. The samples were centrifuged at 3000 x g for 10 minutes. 1ml of ice-cold isopropanol was added to each sample and the mixture was transferred to a column. The samples were centrifuged at 3000 x g for 1 minute. The flow through was removed. 4ml of buffer RWT was added to the column and centrifuged at 3000 x g for 1 minute. The flow through was removed. 2.5ml of buffer RPE was added to the column and centrifuged at 3000 x g for 5 minutes. The column was placed into a clean 15ml collection tube and 200µl of RNAse free water was added to the centre of the column and spun at 3000 x g for 1 minute. 800µl of 100% ethanol was added the supernatant and then vortexed briefly. The mixture was transferred to a column and spun at 8000 x g for 15 seconds. The flow through was discarded. 500µl of buffer RPE was added to the column and it was then centrifuged at 8000 x g for 15 seconds. The flow through was discarded. The column was centrifuged at 8000 x g for 1 minute. The column was placed in a RNAse free 1.5ml microcentrifuge tube and 20µl of RNAse free water was added to the centre of the column. The column was centrifuged at 16000 x g for 1 minute. The

concentration of the purified RNA was measured using a nanodrop and the RNA was stored at -80°C for future use. The RNA was used for RT-PCR.

2.27 - Determining cAMP Concentration

Transfected primary myometrial cells were grown in a 96 well plate at a density of 40000 cells/well for 48 hours. The cells were treated with 100µM of forskolin for 30 minutes. The cAMP levels of the cells were measured using CISBIO cAMP HTFR assay following the manufacturer's instructions. The fluorescence was measured using PHERAstar FS plate reader. The standards were plotted using GraphPad Prism and 4-parameter curve fit was used to measure the concentration of cAMP of test samples.

2.28 - Phospho-MAPK Array Kit

Primary myometrial cells were transfected with either siControl or siPLCL-1 and grown on a 6 well plate. After 48 hours of transfection, 200µl of lysis buffer 6 was added to each well, and the cells were scraped into a 1.5ml microcentrifuge tube using a cell scraper. The lysates were rotated in 4°C for 30 minutes. The samples were centrifuged at 16000 x g for 5 minutes. The supernatant was transferred to a clean 1.5ml microcentrifuge tube. 1.1ml of array buffer 1 and 20µl of antibody detection cocktail was added to each sample and the samples were incubated at room temperature for an hour. 2ml of array buffer 5 was added to each well of the 4 well multi-dish. One membrane was placed inside each well in the multi-well dish and incubated for one hour on a rocking platform shaker at room temperature. Array

buffer 5 was removed from the well dish. The lysate/antibody samples were added to the dish containing the membrane and the membranes were incubated overnight on a rocking platform shaker at 4°C. The membrane was placed in a plastic container, containing 20ml of wash buffer and incubated for 10 minutes at room temperature on a rocking platform shaker. 2ml of Streptavidin-HRP was added to each membrane and incubated for 30 minutes at room temperature on a platform shaker. The membrane was placed in a plastic container, containing 20ml of wash buffer and incubated for 10 minutes at room temperature on a rocking platform shaker. The wash buffer was removed and 1ml of chemi-reagent was added to each membrane and incubated for 1 minute. The membrane was exposed on an autoradiography film cassette and developed in a dark room. The film was visualised on a Syngene chemiluminescence detection machine and used to scan the film. The pixel density of each dot blot was measured using ImageJ.

RNA Sequencing

2.29 - Library Preparation

The total RNA from the transfected primary myometrium samples were used for RNA sequencing. The cDNA was synthesised using TruSeq Stranded mRNA assay. In TruSeq Stranded mRNA, the mRNA was first isolated from 1µg of total RNA using an immobilized poly-oligo(dT) beads. The poly(A)tail of the mRNA binds to immobilized poly-oligo(dT) beads in high salt conditions, removal of salt then allows mRNA to be isolated. The RNA is then fragmented and using random primers the RNA is converted

into a library of cDNA fragments. Adaptors were added to both ends of the cDNA fragments. The cDNA fragments were then amplified using PCR, cDNA was then sequenced using single end high-thoroughput sequencing (Illumina SN300).

2.30- Processing of RNA-sequencing Data

RNA libraries were sequenced using single end high-thoroughput sequencing (Illumina SN300) at SourceBio.Each sequenced read was 100 base pairs in length, the total number of reads for all 12 samples was 280,787,298 and the average number of reads per sample was 23,398,942. Reads from RNA-sequencing data were aligned with the reference genome UCSC h19 GRCh37 using STAR. Samples were quality assessed using FastQC. Reads were normalised in DESeq and transcripts per million(TPM) calculated using R. Patient batch effect was removed using the limma function in R.

2.31 –RNA-sequencing Analysis and Identification of Differentially Expressed Genes

The reads from each gene were analysed using principle component analysis (PCA). PCA is a mathematical model used to reduce the dimensionality of large data sets, based on the variance. The first principle component (PC1) is the direction along the variables whereby there is the most variation. The second principle component (PC2) is the direction where the variables show the second most variation . Each variable

or data set represents the sequence reads of a single gene in a sample. Each gene is then scored on how much influence it has on the length and direction of each PC, this is called loadings. Each sample has mutiple loadings for each gene and this is termed an eigenvector. The loadings are then combined for each sample to get a single value for PC1 and PC2, this is then plotted; samples with similar values will cluster. Therefore, PCA is a technique that is used to assess similarities in variance between different samples and to group similar samples together.

Principle component analysis (PCA) and heat map plotting was conducted in R and the data transformed using regularized-logarithm transformation, with the function being: rld = rlog(dds, blind=FALSE). Heat map plotting was done using Euclidean distance. PCA was done using an unsupervised clustering technique. Differential expression analysis was performed using DESeq2, using the Wald statistical test. Knockdown samples were compared to siControl samples and oxytocin treated samples compared to no-oxytocin samples to identify any differentially expressed genes (DEG), using the Wald statistical test. Genes were identified as DEG if there was a more than a 2-fold change and if the p value was less than 0.05 (fold change>2 and $P \leq 0.05$). Benjamini-Hochberg procedure was applied to control the false discovery rate and the cut-off point was 0.05. KEGG pathway enrichment analysis was performed by Hyper-geometrical statistical test, using the R function 'phyper'. In the KEGG pathway enrichment analysis; down regulated and up-regulated genes were matched with 300 different biological pathways. Using a hyper-geometrical statistical test, pathways with a p value less than 0.05 were thought to be significantly enriched.

2.32 - Software for RNA Sequencing Analysis

Read counts were aligned in STAR. Quality control was done using FASTQC. Patient batch effect was removed using R. Read count normalisation was done in DESeq. Transcripts per million was calculated in R. PCA and heat map analysis was done in R. KEGG enrichment pathway analysis was done in R.

2.33 - Statistical Analysis

The data was evaluated using GraphPad prism 6.0 and Excel. Student's two-sample t test was done to look for significant differences in the means of two independent samples (unpaired), assuming the values follow a normal distribution. A one-way ANOVA test was done to look for significant differences in the means of three or more independent samples (e.g. section 3.2.2). The one-way ANOVA test was followed by a post-hoc test. The post hoc test used was either Tukey's honestly significant difference (HSD) post hoc test or Dunnett's post hoc test. It was assumed that the values followed a normal distribution. A two-way ANOVA test was done to identify any significant difference in the means of samples, when there were two independent variables. For example, in section 4.2.2, the two independent variables were without oxytocin (0 hours) and with oxytocin and treatment time, thus a two-way ANOVA test was conducted. The post hoc test following the two-way ANOVA was either Tukey's honestly significant difference (HSD) post hoc test or Dunnett's post hoc test, and the data was assumed to follow a normal distribution. A *P*-value of ≤0.05 was considered as significant.

Chapter 3

PLCL-1 Reduces IP_3 Mediated Calcium Release in Primary Myometrial Smooth Muscle Cells.

3.1 Introduction

It is well established that a rise in calcium is vital for smooth muscle cell contraction. There is a lot of evidence showing that PLCL-1 inhibits intracellular calcium release. PLCL-1 binds to IP_3 and inhibits IP_3 mediated release of calcium from intracellular stores in COS-1 cells overexpressing PLCL-1 (Kanematsu *et al*, 1992) (Takeuchi *et al*, 2000). Knockdown of PLCL-1 in mice neuronal cells, increases IP_3 mediated release (Harada *et al*, 2005). Treating HESCs with siPLCL-1, led to a reduction in IP_3 mediated calcium release (Muter *et al*, 2016). There is also a lot of evidence showing that PLCL-1 expression is reduced in the labouring myometrium in women compared to women not in labour (Chan *et al*, 2014)(Sharp *et al*, 2016) (Mittal *et al*, 2010). Therefore, it is hypothesised that PLCL-1 expression is reduced during labour to release the inhibition PLCL-1 has on IP_3 mediated calcium release. To partly test this hypothesis, the effect of knocking out and overexpressing PLCL-1 on $[Ca^{2+}]_i$ in primary myometrial cells was investigated.

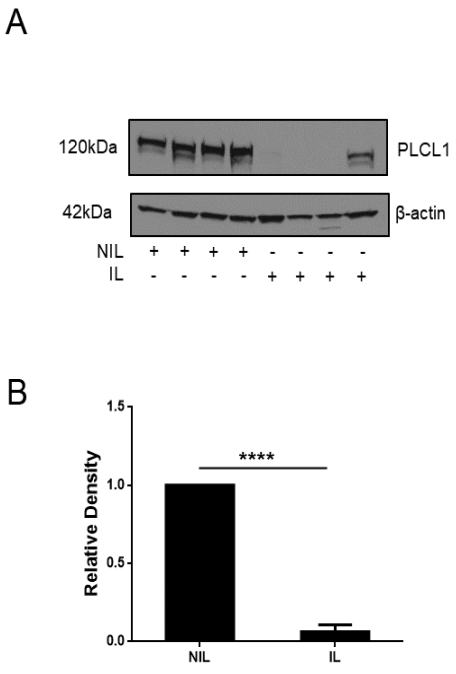
One of the objectives of this project was to create different cell lines with deleted regions of PLCL-1 using CRISPR-Cas9 and to use the cell lines for different assays that helped determine the function of each region. CRISPR-Cas9 requires cells that can be cultured for a few months as a clonal population of cells with the targeted deletion need to be created. Primary myometrial cells are only able to grow until passage 4, thus they are not suitable for CRISPR-Cas9. A cell line derived from myometrial cells would be more suitable for the use in CRISPR-Cas9. A commercially available cell line from uterine myometrium smooth muscle: PHM-41 cells, were first thought to be

suitable, however after culturing PHM-41 cells, it was observed they had a slow doubling rate. Therefore, a robust cell line derived from the myometrium was needed, this new cell line (Myla) required characterisation, to determine if they retained features of primary myometrial cells.

3.2 Results

3.2.1- PLCL-1 Expression Decreases in Myometrium of Labouring Women

Western blotting showed a decrease in PLCL-1 expression in the myometrium of labouring women compared to women not in labour. There was a ~90% decrease in PLCL-1 expression in myometrium of labouring women (Figure 3.2.1).



3.2.1 – ***PLCL-1 Expression in the Myometrium During Labour.*** A) Myometrial biopsies were collected from women not in labour (NIL) and women in labour (IL). The biopsies were mechanically homogenised and then differences in PLCL-1 expression between the two groups were detected using western blotting. B) Western blot band density of IL samples relative to NIL samples. n=4, error bars (standard deviation). Student's t test $P \leq 0.05$ (*). Figure from Dr Paul Brighton.

3.2.2 - Validating siPLCL-1 and PLCL-1 Transfection of Primary MSMCs Using RT-PCR and Western Blotting

Primary MSMCs were transfected either with siControl, siPLCL-1 or PLCL-1 expressing plasmid. The transfection efficiency was ascertained using RT-PCR and western blotting. Knocking down PLCL-1 led to a ~60% reduction in PLCL-1 mRNA and protein compared to control cells. Overexpressing PLCL-1 increased detected PLCL-1 mRNA by 3-4 folds at both the mRNA and protein levels, compared to control (Figure 3.2.2).

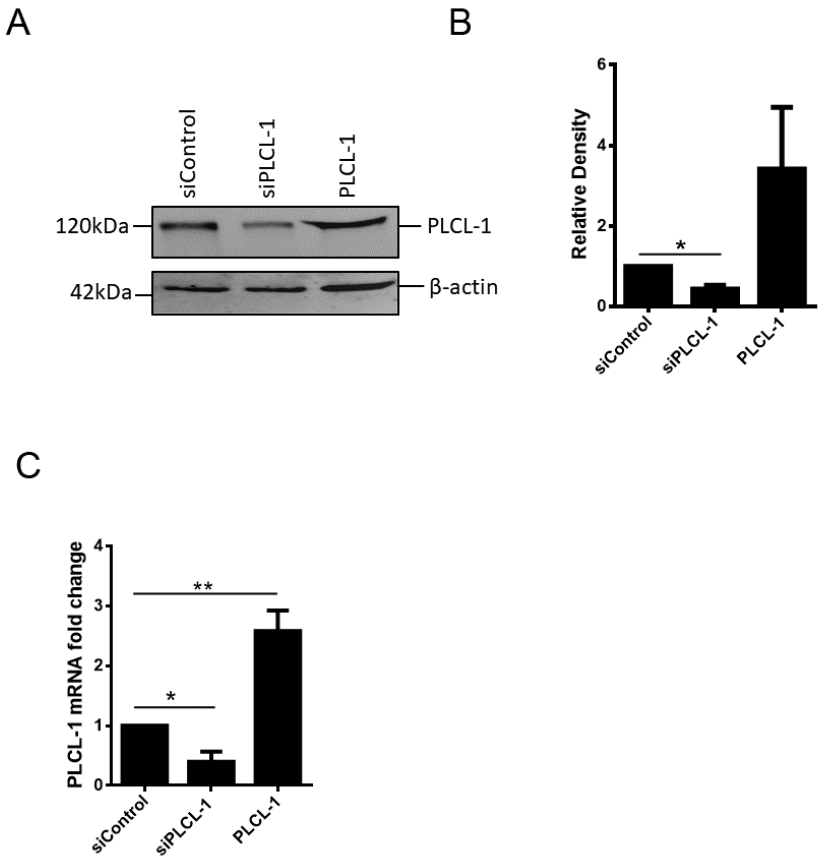


Figure 3.2.2 - ***Validating siPLCL-1 and PLCL-1 Transfections*****.** A) Primary MSMCs were transfected with either siControl, siPLCL-1 or PLCL-1, fold change analysis was done relative to siControl. A) Western blot of transfected cell lysates. B) Western blot band density of siPLCL-1 and PLCL-1 relative to siControl. C) RT-PCR was conducted to investigate the fold change in PLCL-1 mRNA. Data was analysed using one-way ANOVA with Dunnett's post hoc test. $P \leq 0.05$ (*), n=3, error bars represent standard deviation. siPLCL-1 and PLCL-1 samples were compared to siControl.

3.2.3 - Knocking Down PLCL-1 Increases Oxytocin Mediated Increases in $[Ca^{2+}]_i$, Whilst Overexpressing PLCL-1 Supresses Oxytocin Mediated Increases in $[Ca^{2+}]_i$.

Transfected cells were treated with 10nM Oxytocin (OT), and the changes in calcium concentrations were assessed using the calcium indicator fluorescent dye indo-1, and this was done for 600 seconds (Figure 3.2.3b). The experiment was repeated three times. Figure 3.2.3b; panels A-C represent the calcium oscillations observed from one experiment, each line or curve represents the calcium oscillations of one cell. The area under graph is calculated using the trapezoidal method. Trapezoids of equal width are fitted from the baseline under each calcium curve from 0 seconds to 600 seconds. The area of each trapezoid fitted under each curve is calculated. The area under the curve (AUC) is then the sum of the areas of all the trapezoids under the curve (Figure 3.2.3a). Each cell will have its own AUC, because each cell has its own calcium curve. The mean AUC was calculated from all the repeats (n=3).

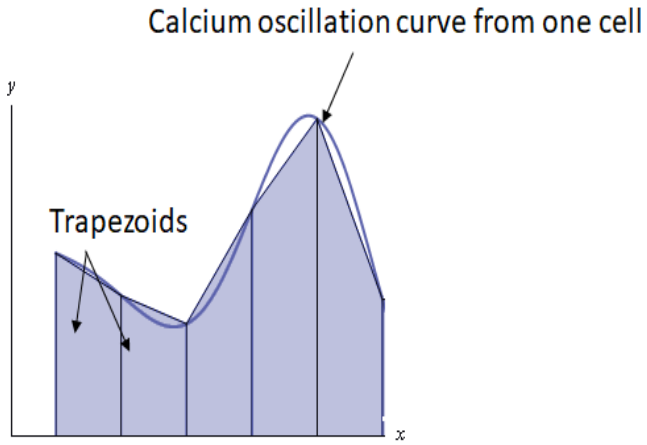


Figure 3.2.3a - ***Schematic Representation of how the Area Under a Calcium Curve is Calculated*** Trapezoids are fitted under the calcium curve. The area of each trapezoid is calculated. The sum of the areas of all the trapezoid under the curve is then used as an approximation for the area under the curve.

Treatment with siPLCL-1 increased the number of oscillations upon stimulation with OT, compared to control (Figure 3.2.3b). Overexpressing PLCL-1 decreased the oscillation frequency compared to control. The maximum peak response in siControl cells was ~2, this increased to ~4 when PLCL-1 was knocked down and decreased to ~1.5 when PLCL-1 was overexpressed. The mean area under the curve (AUC) increased from 107 (SD ± 28) to 382(SD ±125) in siControl and siPLCL-1 cells, respectively. Overexpressing PLCL-1 reduced the mean AUC to 69 (SD ± 12) in comparison to control cells (Figure 3.2.3b). The increase in the oscillation frequency, maximum peak response and AUC when PLCL-1 was knocked down, suggests that PLCL-1 decreases oxytocin mediated increase in intracellular calcium concentration.

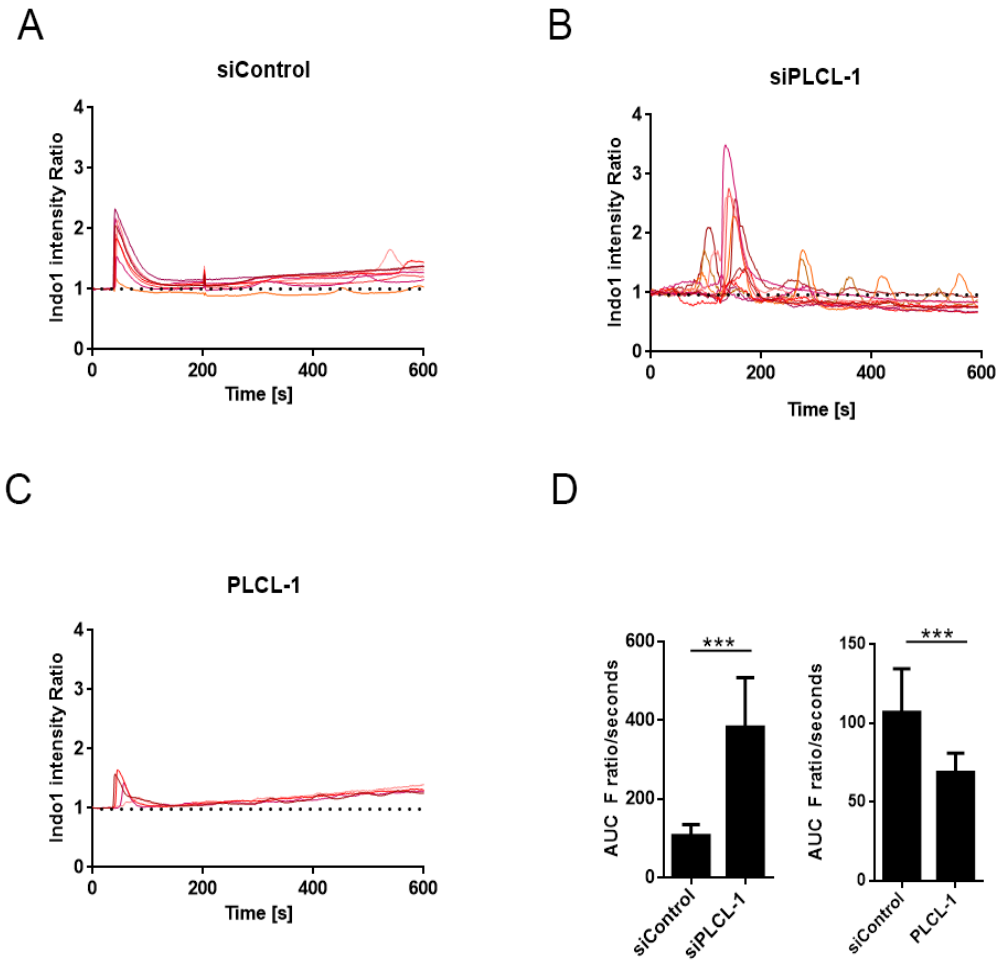


Figure 3.2.3b - ***Effect of siPLCL-1 and PLCL-1 on*** $[Ca^{2+}]_i$. A-C) Primary myometrial cells were transfected with siControl, siPLCL-1 or 1µg PLCL-1 and then stained with indo1-am and treated with 10nM oxytocin. The cells were excited at 345nm and fluorescent emission was observed at 475nm and 400nm, the ratio between the two emission spectra were plotted against time. Dotted line represents the baseline, these graphs are from one experiment that is an accurate representative of all the repeats, which were 3. D) Graphs demonstrating the mean area under the curve (AUC) from the baseline, this is the mean AUC for all the repeats, thus n= 3. Error bars (standard deviation). Student's t test $P \leq 0.05$ (*).

3.2.4- Characterisation of a New Myometrial Cell Line (Myla cells)

There was a need for a robust myometrial cell line that has a fast doubling rate, for efficient CRISPR-Cas9 vector transfection; to create a cell line that had deleted PLCL-1 regions. Therefore, primary myometrial cells were transfected with a temperature sensitive simian virus 40 (SV40) T antigen, to induce immortalisation. The immortalisation was done by Dr Anne Straube (University of Warwick). When the cells are incubated at a temperature of 33°C, the cells have a phenotype close to the wild-type SV40 T antigen-immortalized cells; with a fast doubling rate. However, when the cells are transferred to restrictive growth temperature at 37°C, the cells revert to having a morphology that is similar to primary MSMCs. Primary MSMCs have a spindle like morphology with an elongated shape and nucleus, this is similar to Myla cells grown at 37°C. However, the cells grown at 33°C do not have an elongated nucleus, but rather an oval shaped nucleus (Figure 3.2.4a).

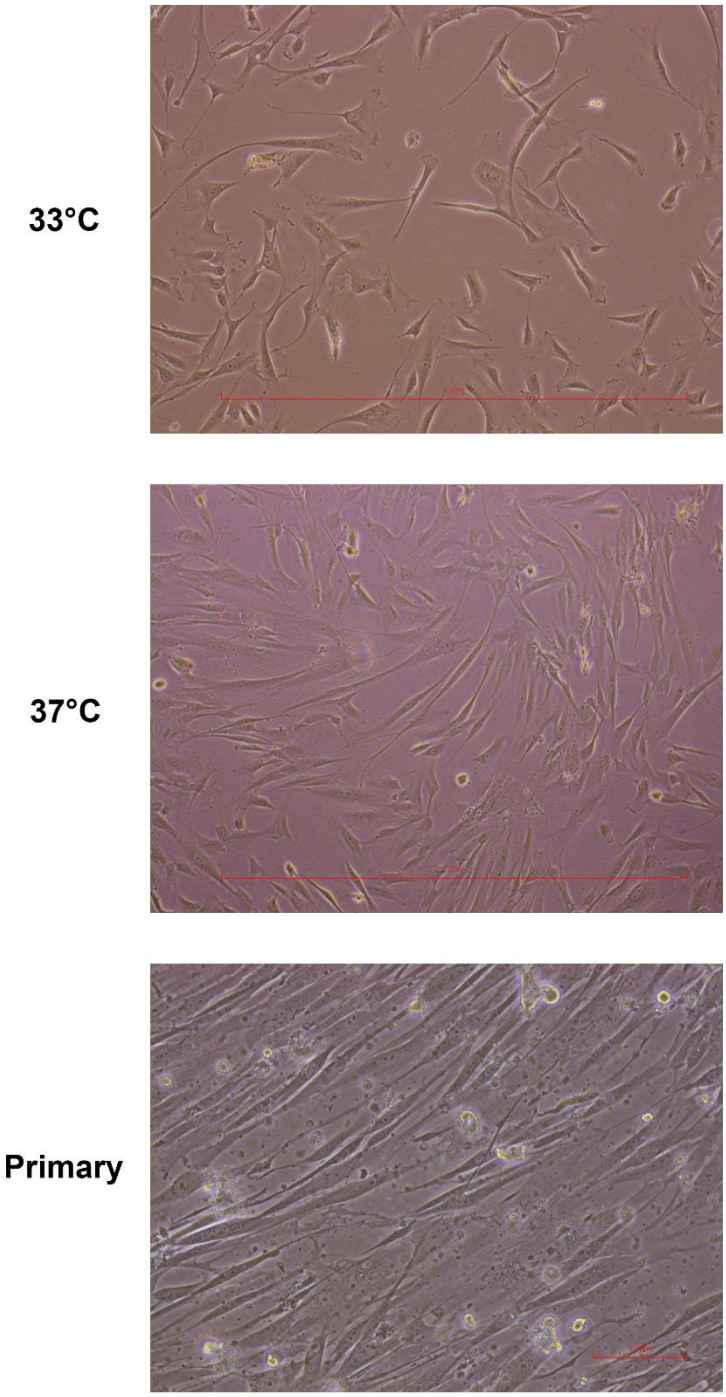


Figure 3.2.4a – *Phase Contrast Image of Primary MSMCs and Myla cells*. Phase contrast images were taken from cells Myla cells grown at 33°C and 37°C and also primary MSMCs grown at 37°C.

Myla cells were loaded with the calcium indicator dye fluo4-am and treated with 10nM OT for 10 minutes. There was a visibly larger maximal response to OT in cells grown at 37°C compared to those grown at 33°C, with a fluo4 intensity ratio of 8 compared to 1.5, respectively. Additionally, the AUC of Myla cells grown at 37°C was greater compared to cells at 33°C (143 and 111, respectively). However, there were no oscillations observed in both 33°C and 37°C cells (Figure 3.2.4b)

RT-PCR analysis done on Myla cells at 37°C showed a 50% increase in desmin mRNA levels and a 20% decrease in vimentin mRNA, compared to cells grown at 33°C (Figure 3.2.4c). Desmin is a marker for smooth muscle cells, whilst vimentin is a marker for fibroblast cells (Capetanaki *et al*, 1997) (Owens, 1995) (Strutz *et al*, 1995). The RT-PCR results show that the Myla cells change from having a fibroblast phenotype at 33°C to having a more smooth muscle cell characteristics after they are transferred to 37°C, this may explain why the Myla cells grown at 37°C have a larger calcium response to OT.

RT-PCR analysis also showed that Myla cells expressed OTR, PGFTR and CACNA1C (Calcium Voltage-Gated Channel Subunit Alpha1 C). There was a significant increase in OTR and PGFTR mRNA in the 37°C cells, a 50% and 300% increase, respectively (Figure 3.2.4c). There was no significant difference in expression of CACNA1C mRNA in the cells grown at 33°C and 37°C.

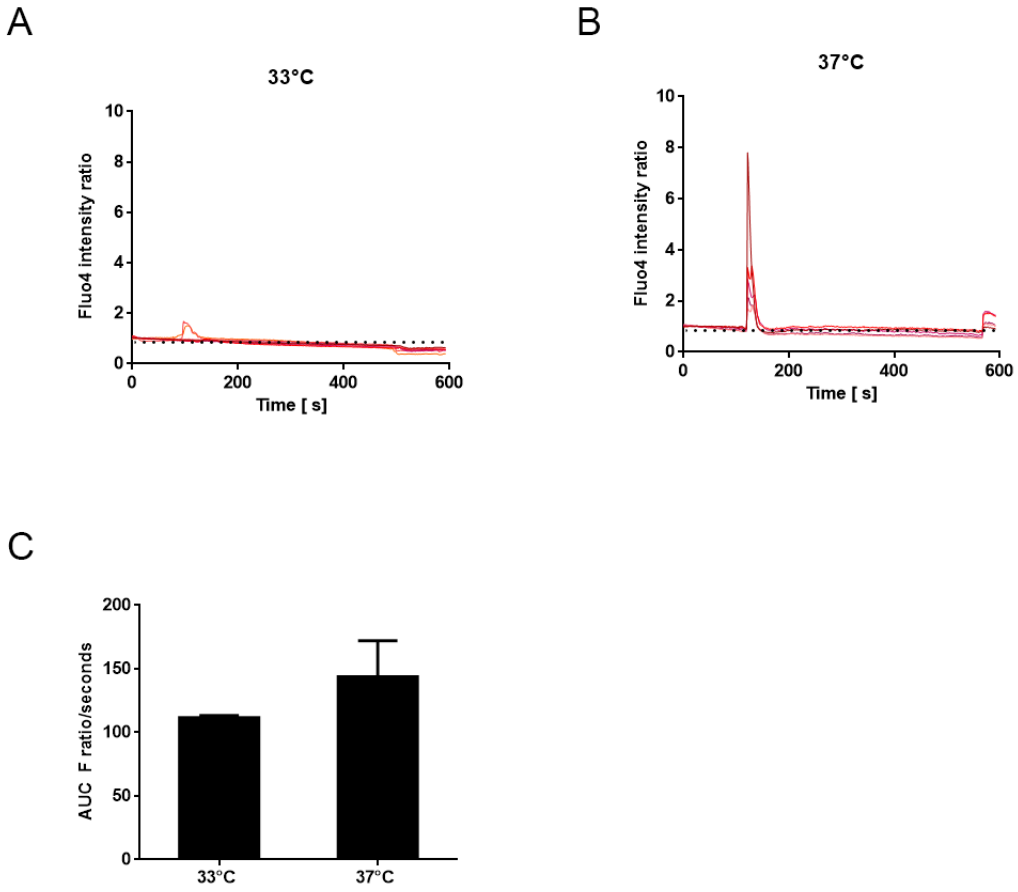


Figure 3.2.4b- ***Response of Myla cells to 10nM Oxytocin.*** A&B) Myla cells stained with fluo4am and treated with 10nM oxytocin. The ratio between the basal fluorescent signal before the addition of OT and the fluorescent signal after addition of OT was calculated. Dotted line represents the baseline. These graphs are from one experiment that is an accurate representative of all the repeats, which were 3 C) Graph demonstrating the average area under the graph, which was calculated from all the repeats, n=3, error bars (standard deviation). Student's t test $P \leq 0.05$ (*).

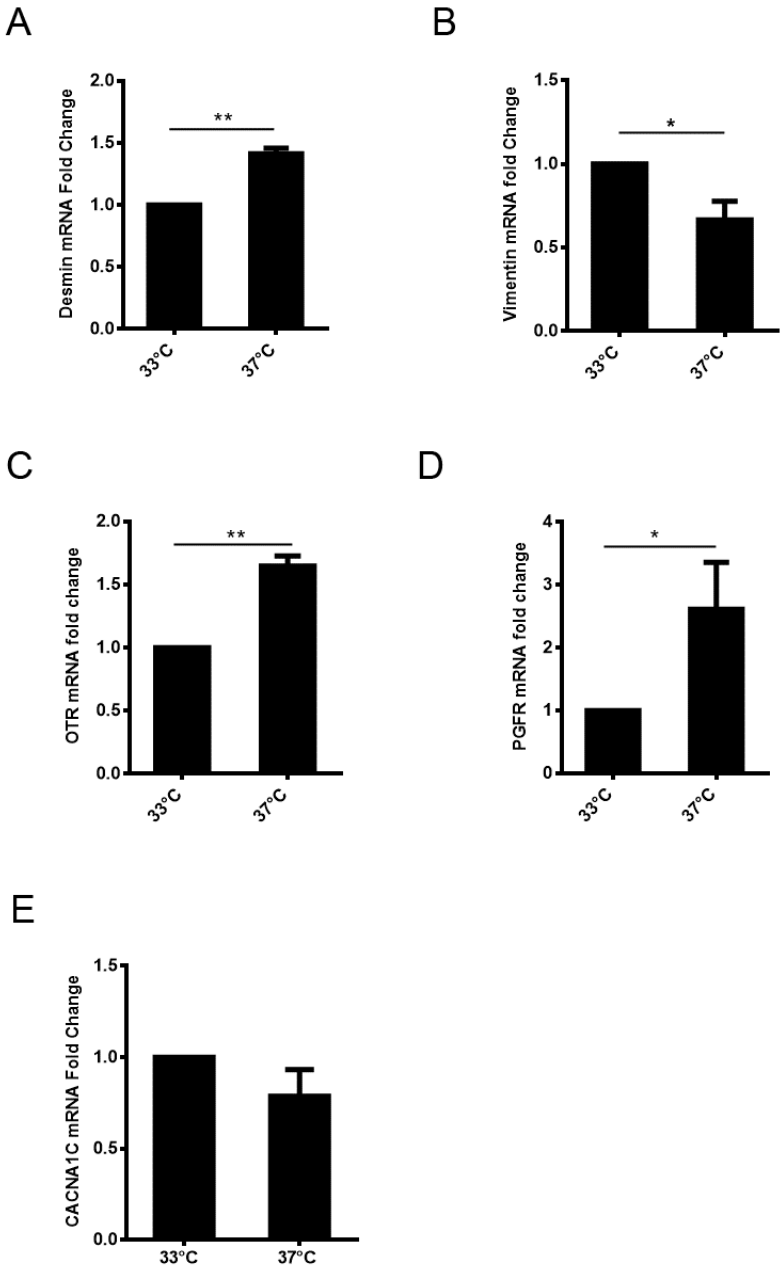


Figure 3.2.4c- *Characterisation of Myla cells*. Total RNA was extracted from Myla cells, and converted to cDNA for RT-PCR. 5 genes were investigated for changes in their mRNA upon a change in growth temperature : A) Desmin, B) Vimentin, C) Oxytocin receptor (OTR), D) Prostaglandin F2α receptors (PGFR) and E) CACNA1c. Data was analysed using Student's t-test, $P \leq 0.05$ (*), n=3, error bars represent standard deviation.

Myla cells were stained for desmin and vimentin (Figure 3.2.4d). Myla cells showed a visible decrease in staining for vimentin when grown at 37°C, however there was no notable difference in desmin staining. Staining for CACNA1C showed that the Myla cells and primary MSMCs both expressed CACNA1C and there was no notable difference in the staining between the primary cells and the cell line (Figure 3.2.4e).

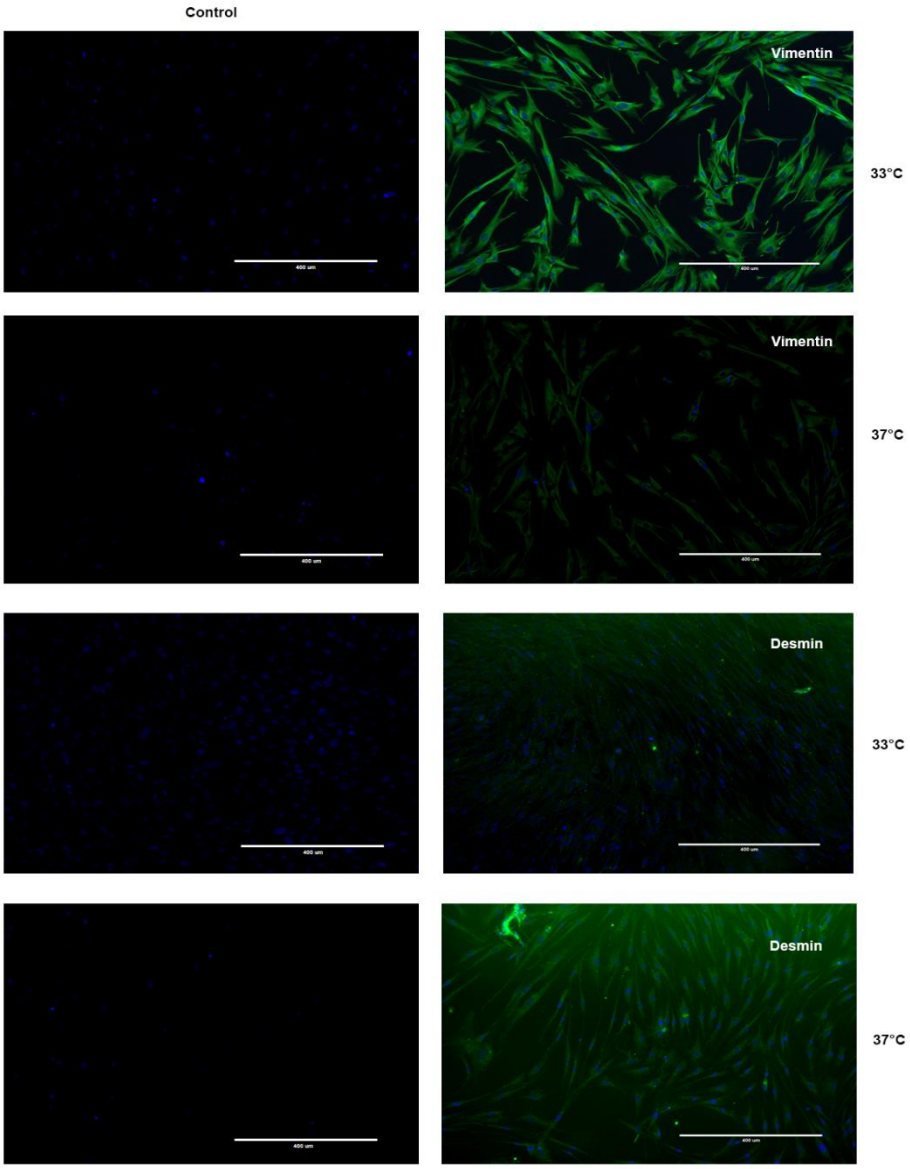


Figure 3.2.4d- ***Investigating the Phenotype of Myla Cells***. Myla cells were grown at 33°C and 37°C and were then stained for vimentin and desmin. The control cells were not exposed to primary antibodies, only to the secondary antibody. n=3.

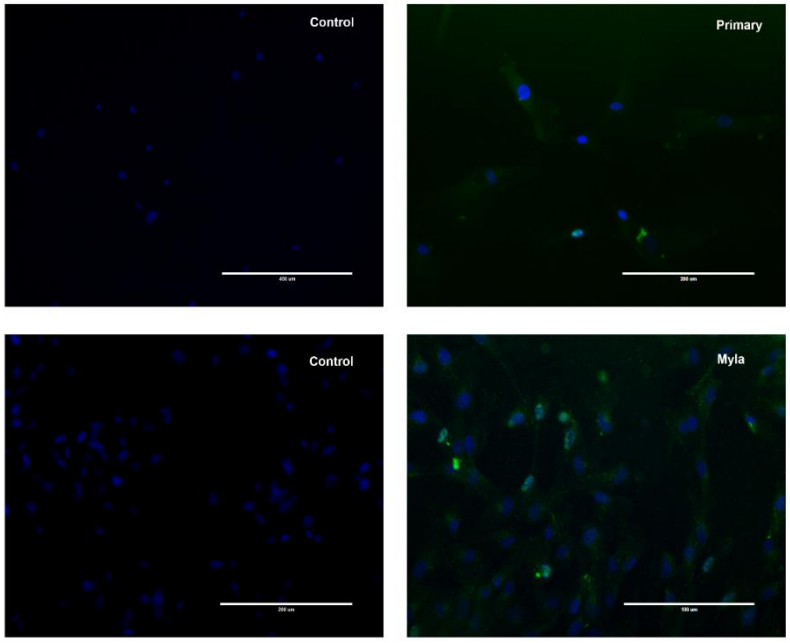


Figure 3.2.4e- ***Staining Myla Cells Grown at 37°C and Primary MSMCs for CACNA1c.*** Myla cells were grown at 37°C and primary MSMCs were stained using a CACNA1c antibody. The control cells were not exposed to primary antibodies, only to the secondary antibody. n=3

3.3 Discussion

PLCL-1 Expression in the Myometrium Decreases During Labour

A consistent reduction in the expression of PLCL-1 in the myometrium of women during labour has been observed in many different studies. RNA sequencing done to compare differences in gene expression between non-labouring and labouring myometrium in humans, showed a reduction in PLCL-1 expression during labour (Chan *et al*, 2014) (Sharp *et al*, 2016). Comparably to the human studies; RNA

sequencing done on myometrium from mice, also showed a reduction in PLCL-1 during labour (Migale *et al*, 2016). Similarly, to the RNA sequencing studies, microarrays studies done on the human myometrium showed a downregulation of PLCL-1 during labour (Bethin *et al*, 2003) (Esplin *et al,* 2005) (Mittal *et al*, 2010). The result from section 3.2.1 was concurrent with the previous studies; showing a reduction in PLCL-1 expression during labour.

Preceding studies done on neuronal cells, COS-1 cells and HESCs have shown that PLCL-1 inhibits the release of calcium from intracellular stores, this effect was also observed in primary myometrial cells (Takeuchi *et al*, 2000) (Harada *et al*, 2005) (Muter *et al*, 2016). PLCL-1 was shown to cause a reduction in oxytocin induced increases in intracellular calcium concentration in the myometrium (Figure 3.2.2b). Oxytocin is an important peptide hormone during the stimulation phase of pregnancy and oxytocin signalling leads to a release of calcium from intracellular stores (Section 1.7.3). A rise in calcium is crucial for contraction to occur in the myometrium. The downregulation of PLCL-1 during labour (Figure 3.2.1) may be a mechanism to lessen the inhibitory effect that PLCL-1 has on the release of calcium by CAPs such as oxytocin (Figure 3.2.2b). The downregulation of PLCL-1 may lead to an increase in the contraction frequency during labour, thus it may be that PLCL-1 levels in the myometrium are kept high during the quiescent phase of pregnancy to dampen the likelihood of contractions. However once full term is reached, PLCL-1 levels are reduced leading to an increase in intracellular calcium concentration, which increases the likelihood of contractions occurring.

Proposed Mechanism for PLCL-1 Inhibiting IP_3 Mediated Calcium Release.

PLCL-1 was shown to decrease oxytocin induced $[Ca^{2+}]_i$ in MSMCs, and this result validated previous data, which showed the same effect of PLCL-1 on $[Ca^{2+}]_i$ (Takeuchi *et al*, 2000) (Harada *et al*, 2005) (Muter *et al*, 2016). One explanation for the inhibitory effects of PLCL-1 on rises in $[Ca^{2+}]_i$ is that PLCL-1 binds to IP_3, to sequester it , which physically prevents it from binding to IP_3 receptors on the ER (Figure 3.3.1).

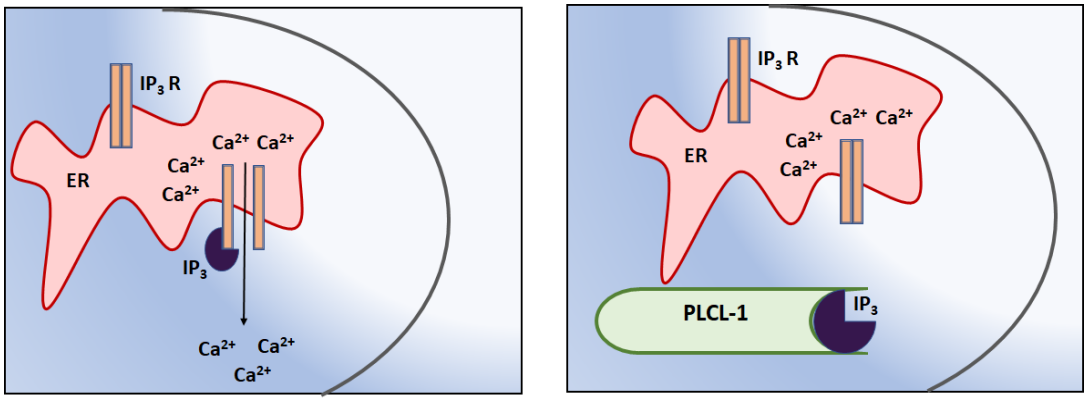


Figure 3.3.1- ***Schematic Representation of PLCL-1 Sequestering IP_3***. MSMC on the left shows the IP_3 being able to bind to IP_3R on endoplasmic reticulum (ER) without PLCL-1 present. The presence of PLCL-1 (right) means IP_3 is sequestered and not able to bind to IP_3R, thus there is no release of calcium from the ER.

Characterisation of Myla cells

Transferring the Myla cells from 33°C to 37°C led them to having characteristics that are associated with primary myocytes. The Myla cells changed their morphology from being more fibroblast like at 33°C to being more smooth muscle like at 37°C.

The Myla cells went from have an oval shape to becoming more elongated and having more contact with adjacent cells, this was similar to primary MSMCs (Figure 3.2.4a).

At 37°C the Myla cells had a larger response to oxytocin compared to cells grown at 33°C, the larger calcium spike at 37 suggest that the Myla cells are more similar to primary cells in their response to oxytocin. However, both cells grown at 33°C and 37°C showed no calcium oscillation, this is in contrast to primary MSMCs.

The RT-PCR analysis suggested that Myla cells at 37°C had gene expression profile that is similar to smooth muscle cells, as there was a decrease in vimentin and an increase in desmin (Capetanaki *et al*, 1997) (Owens, 1995) (Strutz *et al*, 1995). The staining for vimentin validated the RT-PCR results, however the desmin staining showed no difference in staining between 33°C and 37°C. The RT-PCR showed an increase in OTR expression, which may explain why there was a larger calcium response upon treatment with oxytocin.

The CACNA1c gene codes for a subunit for a subunit of the L-VOCC, and it was found to be expressed in Myla (Qin *et al*, 2002). Both the primary MSMCs and the Myla cells stained for CACNA1c.

Although Myla cells appear to have a morphology similar to primary MSMCs and gene expression similar to smooth muscle cells, there are limitations to the RT-PCR results. The RT-PCR results would have been more robust if the same experiment was repeated with primary MSMCs, which would have shown if the expression of desmin and vimentin in Myla cells were similar to primary cells, and not just that the gene expression changed when the cells were transferred to 37°C.

Chapter 4

Analysis of Transcriptome Changes After PLCL-1 Knockdown by High-throughput RNA-sequencing

4.1 Introduction

Calcium has been shown to regulate transcriptional expression in smooth muscle cells and other cell types by activating or repressing calcium sensitive transcription factors (Table 4.1.1) The transcriptional regulation by calcium is called excitation-transcription coupling, it transforms temporary calcium signalling and contractile periods into long-lasting regulation of the smooth muscle cell transcriptome.

Table 4.1.1- Examples of Calcium Regulating Transcriptional Changes

Cell Type	Transcription factor/cofactor	Activation or Repression	Affected Genes	Signalling Pathway	References
Vascular smooth muscle cells	Myocardin Serum Response Factor (SRF)	+ + +	Smooth Muscle myosin heavy chain Smooth muscle α-actin c-fos	Activation of RhoA/Rho kinase cascade	Wamhoff *et al*, 2004
Guinea Pig gallbladder smooth muscle cells	cAMP response element binding protein (CREB)	+	Transient receptor potential protein family c (TRPC)	Activation of Ca^{2+}/calmodulin kinase (CaMK)	Morales *et al*, 2007
Vascular smooth muscles from cerebral arteries	cAMP response element binding protein (CREB)	+	c-fos	Activation of Ca^{2+}/calmodulin kinase (CaMK)	Cartin *et al*, 2000
Vascular smooth muscle cells	NFATc3	-	Voltage dependent potassium channel (Kv2.1)		Nieves-Cintron *et al,* 2008 Amberg *et* al, 2004

Any protein that changes intracellular calcium levels may indirectly influence the transcriptome. PLCL-1 has an effect on $[Ca^{2+}]_i$ (Chapter 3), thus PLCL-1 may have transcriptional control in the myometrium. PLCL-1 expression being downregulated in the myometrium of women who are in labour suggests PLCL-1 may be a pro-quiescent gene and its reduction is needed for a successful labour (Chan *et al*, 2014) (Sharp *et al*, 2016). Therefore, it was hypothesised that PLCL-1 may function as a pro-quiescent gene by reducing the expression of CAPS and inducing expression of pro-quiescent genes, this function by PLCL-1 may be mediated through its actions on $[Ca^{2+}]_i$. The hypothesis was tested by knocking down PLCL-1 and observing changes in the transcriptome using RNA-sequencing. The ability of PLCL-1 to increase expression of pro-quiescent genes or decrease CAPs was examined. RNA-sequencing was conducted on cells treated with oxytocin. The difference between the transcriptome of cells with or without OT was studied, and the ability of PLCL-1 to modulate this difference was investigated. The aim of these experiments is to investigate if OT can induce expression of CAPS, and if PLCL-1 can reduce this OT mediated expression.

4.2 Results

4.2.1- Validating PLCL-1 Knockdown Through RT-PCR and Western Blotting

Protein levels of PLCL-1 was reduced in MSMC by siPLCL-1. A scrambled non-targeting siRNA (siControl) was used as a control. Optimum knockdown of PLCL-1 was achieved after treatment with siPLCL-1 for 48 hours, which led to a ~75% decrease in

the expression of PLCL-1 (Figure 4.2.1). The primary cells were treated with either siControl or siPLCL-1 for 48 hours before harvesting for total RNA. The effectiveness of the knockdown treatment was scrutinised using qPCR and western blotting (Figure 4.2.1). Treatment with siPLCL-1 saw a 60% decrease in mRNA and protein levels PLCL-1. The total RNA purity and quality was tested using a bioanalyzer, the total RNA was then used for RNA sequencing analysis.

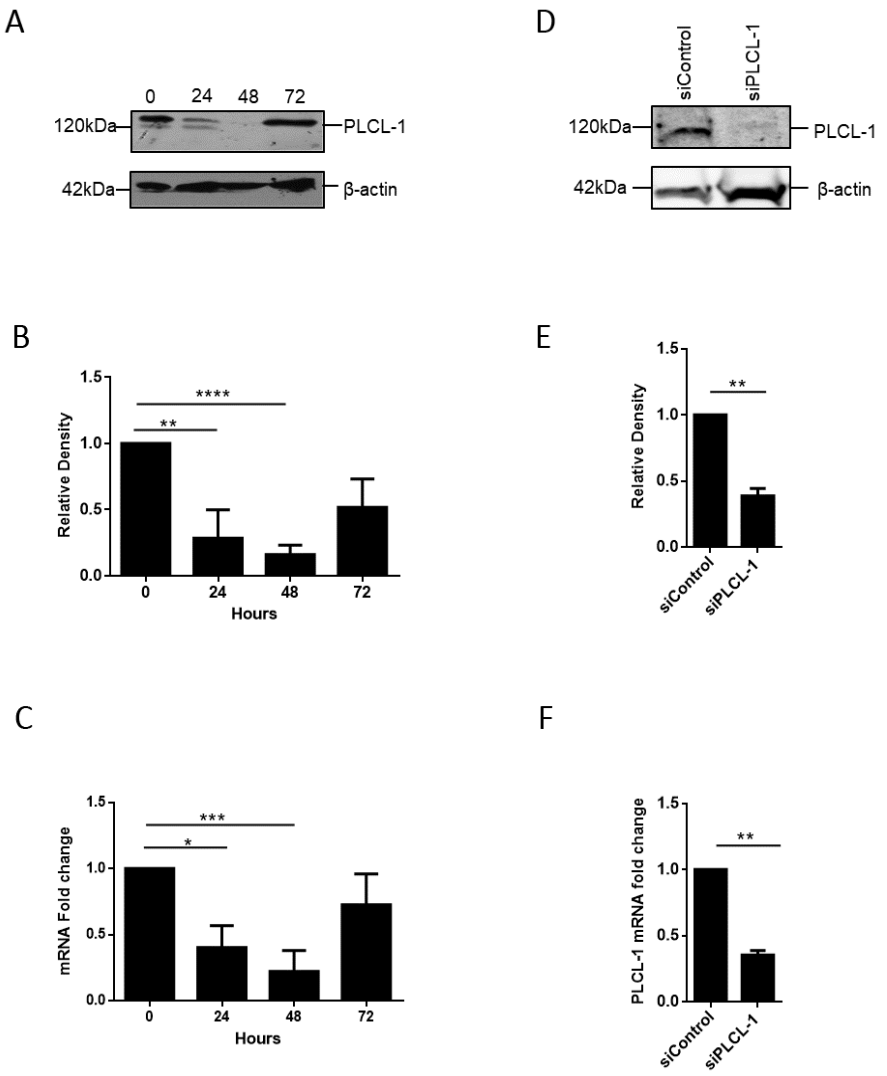


Figure 4.2.1-*Validating Knockdown of PLCL-1 for RNA Sequencing*. Primary MSMCs were transfected with siPLCL-1 for 0, 24, 48 or 72 hours A) Western blot for PLCL-1 expression using lysates that were treated with siPLCL-1 at different time points. B) Relative density of western blot (a), the fold change in PLCL-1 expression was calculated relative to 0 hours. C) RT-PCR was done to investigate PLCL-1 mRNA fold change of each time point relative to 0 hours. D) Cells were transfected with siPLCL-1 or siControl, western blot of transfected lysates. E) Western blot band density of siPLCL-1 relative to siControl. F) RT-PCR was conducted to investigate the fold change in siPLCL-1 mRNA, relative to siControl. Data from panels B and C were analysed using one-way ANOVA followed by Dunnett's post hoc test. Data from panels E and F were analysed using Student's t-test. *P* ≤0.05 (*), n=3, error bars represent standard deviation.

4.2.2- Investigating the Optimal Transcriptional Response to OT

Twelve samples were compared; 6 siControl and 6 siPLCL-1 treated samples, in addition 3 of each group were treated with 10nM oxytocin. The cells were treated with oxytocin to examine the effects of knocking down PLCL-1 on the transcriptome when the cells were treated with a pro-contractile molecule. Optimal treatment time with oxytocin was chosen by the maximal induction of a downstream target of oxytocin, Cyclooxygenase 2 (COX2) (Figure 4.2.2). The primary myometrial cells were treated with 10nm oxytocin for 0 hours, 1.5 hours, 3 hours, 6 hours and 24 hours. The induction of COX2 was measured using qPCR and western blotting. Treatment with oxytocin for 1.5 hours showed the highest induction of COX2 mRNA levels, with a 3-fold induction, this rise dissipated at 3 hours and 6 hours, although an increase in COX2 mRNA levels (~2 fold) was still seen relative to no oxytocin treatment (0 hours). The increase in COX2 mRNA levels decreased after 24 hours of oxytocin to levels similar at 0 hours. The same pattern was observed at the protein level, COX2 levels increased from 1.5 hours to 6 hours, and this increase was then lost after 24 hours. Therefore, the time point chosen for oxytocin treatment for RNA sequencing was 1.5 hours.

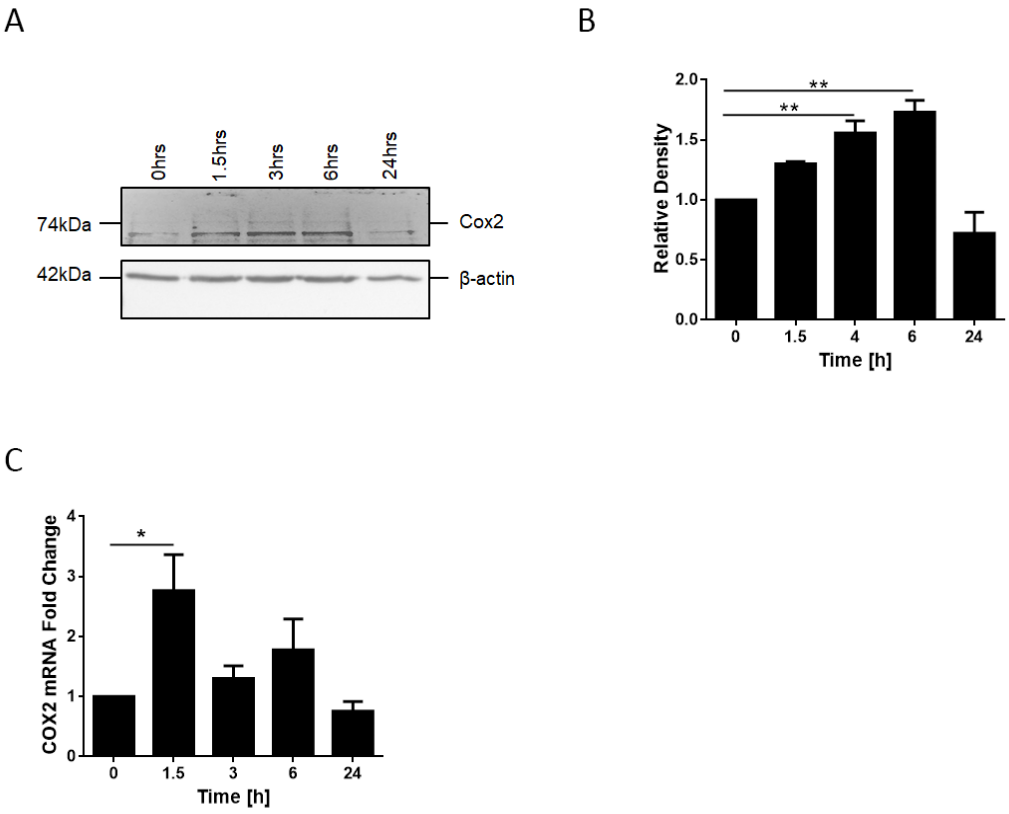


Figure 4.2.2- ***Investigating the Optimal Transcriptional Response to OT*****.** A) Primary myometrial cells were grown until confluent and thereafter serum starved for 24 hours. The cells were then treated with 10nM of OT for 0, 1.5, 4, 6 and 24 hours. Cells were harvested and changes in translation of COX-2 after treatment with OT were investigated using western blotting. B) Relative density of band intensity from western blot (A) was calculated and relative to 0 hours treatment time. C) COX-2 levels were measured using RT-PCR. Data was analysed using two-way ANOVA with Dunnett's post-hoc test , $P \leq 0.05$ (*), n=3, error bars represent standard deviation.

4.2.3- Analysis of RNA sequencing data

In order to investigate how the samples, which are based on comparing oxytocin treatments with no oxytocin treatment and knockdown with control, are grouped together, principal component analysis (PCA) was performed as an unsupervised clustering technique. Samples can be grouped either using supervised or unsupervised learning approaches. In a supervised learning approach, for example in KMeans clustering, the model creates a fixed number of subgroups. This information is known in advance. Whereas in an unsupervised clustering approach, the number of clusters is unknown. The model decides the subgrouping. As an unbiased subgrouping the samples is desired, an unsupervised clustering technique is used. In PCA analysis several principal components are identified that summarise the data by explaining the maximum variance in the data. In Figure 4.3.2a, principal component 1 and 2 were plotted together, which represent 77% of the variation in the data. Based on principal component 1, it is observed that the samples are grouped together depending upon on if they are treated with or without oxytocin. The effect of knockdown on clustering is not substantial, which is why both control and knockdown samples are grouped together.

PC1-PC9 were analysed for the gene reads of siControl and siPLCL-1. In PC1 and PC2 the samples did not cluster together after knockdown of PLCL-1. There was a clear separation between samples that were treated with oxytocin versus those that were not. PC3-PC8 did not show a clustering of samples based on knockdown of PLCL-1. PC9 did show a separation between siControl and siPLCL-1 samples. The genes which

had the most influence on PC9 were noted, and PLCL-1 was found to have the largest influence on PC9 (Figure 4.2.3b) (Appendix 1).

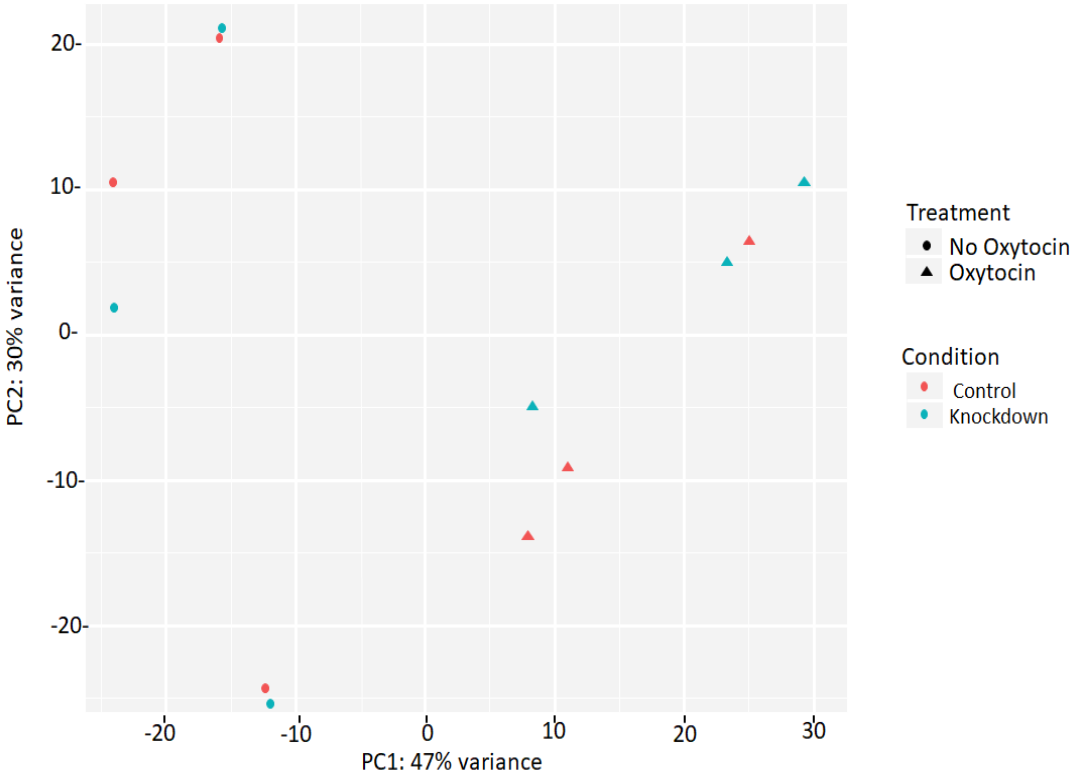


Figure 4.2.3a- ***Principle Component 1 Analysis.*** Primary myometrium cells from 6 different biopsies were transfected and total RNA used for RNA sequencing. Plot of principle component 1 and 2 (PC1, PC2), comparing siControl and siPLCL-1(knockdown), with or without oxytocin. The siControl samples are highlighted in red. The siPLCL-1 samples are highlighted in green. The circles represent the samples that have not been treated with oxytocin. The triangles represent the samples that have been treated with oxytocin. There are 12 samples all together: 3x siControl no oxytocin, 3x siPLCL-1 no oxytocin, 3x siControl with oxytocin and 3x siPLCL-1 with oxytocin.

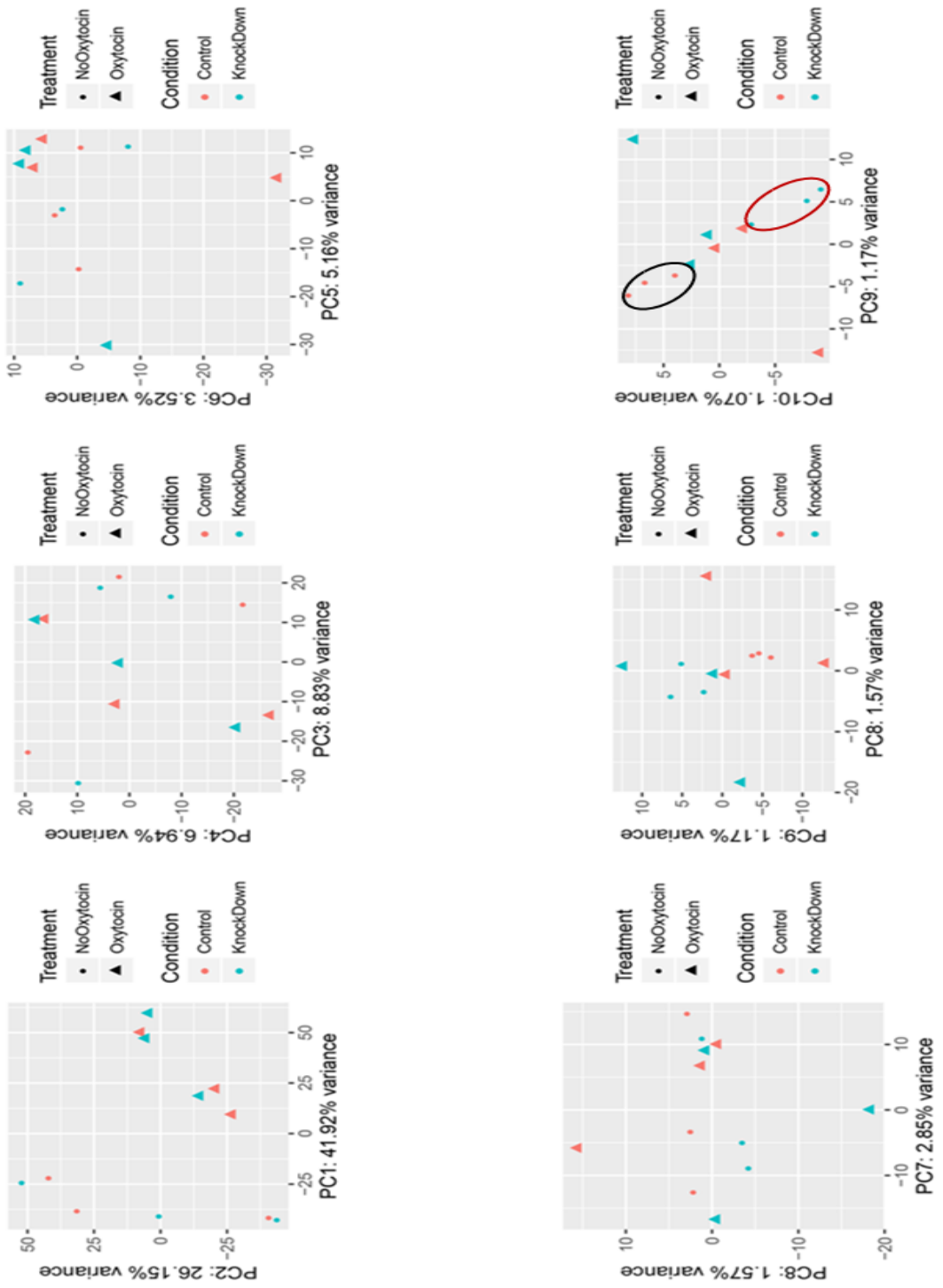


Figure 4.2.3b- ***Principle Component Analysis Plots PC1-PC10***. Primary myometrium cells from 6 different biopsies were transfected and total RNA used for RNA sequencing. Plot of principle component PC1-PC10. comparing siControl and siPLCL-1(knockdown), with or without oxytocin.

Differential expression analysis was performed using R package DESeq2. Based on the Wald statistical test, gene expression values from knockdown and control were compared and a p-value is assigned to each gene. For each gene, a mean expression value was calculated based on all replicates of each condition. The fold change of knockdown relative to control was calculated by dividing the mean expression value of all the replicates of knockdown by the mean expression value of all replicates of control. This was done for each gene in each comparative group; knockdown was compared to siControl and oxytocin treated was compared to no oxytocin treated. This was not done on an individual patient level, rather it was done on the mean values of different conditions.

Gene clusters were assessed by closely looking at the clusters. In Figure 4.2.3a, it was observed that all samples that were treated with oxytocin clustered together to one side of the plot whereas untreated samples cluster to the opposite side. This shows that the samples are clearly grouped together depending upon oxytocin treatment. However, there is no such distinct clustering observed due to knockdown in each patient, except for one, both control and knockdown sample are grouped together.

There were no differentially expressed gene due to knockdown of PLCL-1 experiment (fold change>2 and p-value<0.05). Therefore, patterns in gene expression were explored. To find any patterns in gene expression due to knockdown experiment, the reads for the siPLCL-1 samples were divided by reads from siControl. Fold change was calculated to look at which genes were either up or down regulated when PLCL-1 was

knocked down compared to control; using this criterion, in total 2241 genes are either up or downregulated (fold change >2) in at least one patient. There was no pattern in gene expression according to patients. No visible major gene cluster which was either up or down regulated in all patients was found. To better understand the expression a binary matrix was created; fold change>1 (log fold>0) = 1; fold change<1 (log fold<0) = -1. This will explain which gene is either up or down irrespective of fold change, this method identified the same 2241 genes, which were previously identified (Figure 4.2.3c panel A). Each patient had a different response to different conditions. The main aim in the analysis shown figure 4.2.3.c panel B was to investigate the qualitative response to oxytocin treatment and knockdown of PLCL-1. Therefore, a very simple binary approach was used whereby it was demonstrated how genes are either downregulated or upregulated in different patients, ignoring the scale of change. Some patients might have several fold changes in expression value whereas others might have only a small change though that might be crucial. Very strong changes in some gene expression values in a patient will completely change the cluster. Therefore, a binary approach was used.

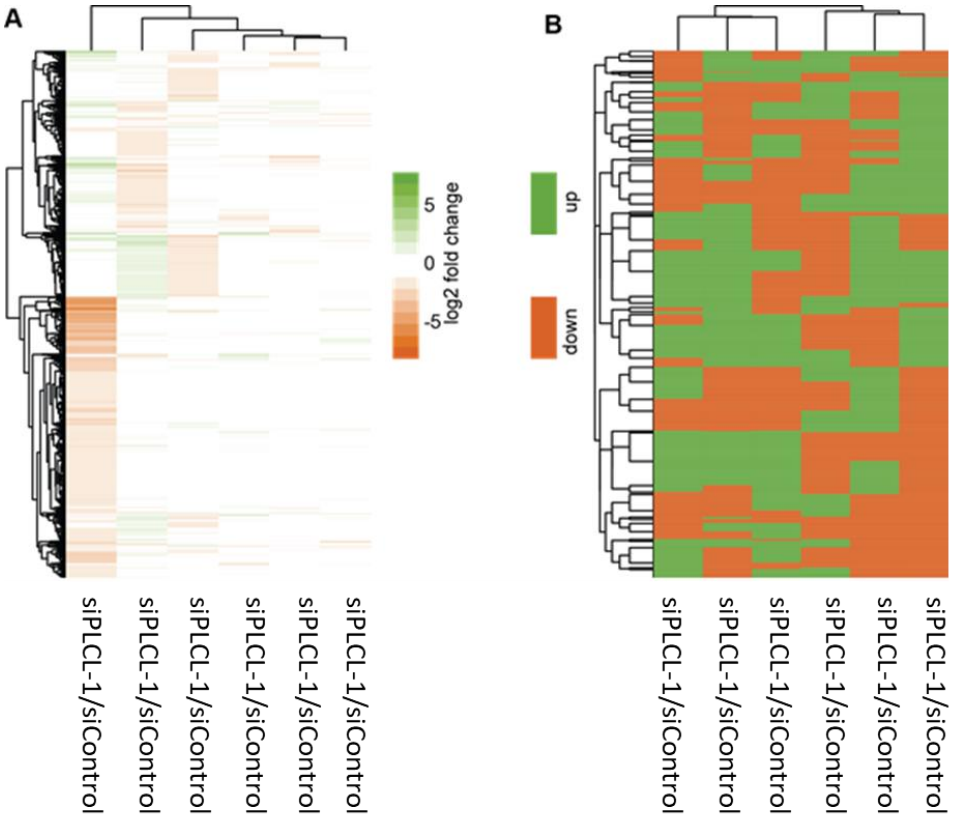


Figure 4.2.3c- ***Heat Map Illustrating Pattern of Gene Expression Due to PLCL-1 Knockdown***. Hierarchical clustering analysis was done on siControl samples treated with or without oxytocin. A) In order to find a pattern of gene expression due to knockdown of PLCL-1; fold change was calculated by dividing siPLCL-1 samples by siControl samples from each patient. There are 6 patients thus there are 6-fold change values, each patient had two samples, one treated with siControl and the other siPLCL-1. Colours represent log fold change, white is between -1 to 1 or without log it will be within fold change 2. Colour between 1 to 2 or -1 to -2 reflect fold change between 2 to 4. B) Gene expression pattern was visualised using a binary matrix; fold change>1 (log fold>0) = 1; fold change<1 (log fold<0) = -1. Plot showing changes in gene expression either up or down irrespective of fold change.

A KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway was used to predict the biological pathways the 2241 genes are involved in (Figure 4.2.3d). Kegg pathway enrichment analysis was performed by Hyper-geometrical statistical test; R function 'phyper' was used for this, and siControl samples were compared with siPLCL-1 samples. According to the Kegg pathway, genes involved in the cell cycle, DNA replication and the TNF pathway were downregulated when PLCL-1 was knocked down. Genes coding for cell adhesion proteins and PI3-AKT pathway were upregulated after PLCL-1 knockdown.

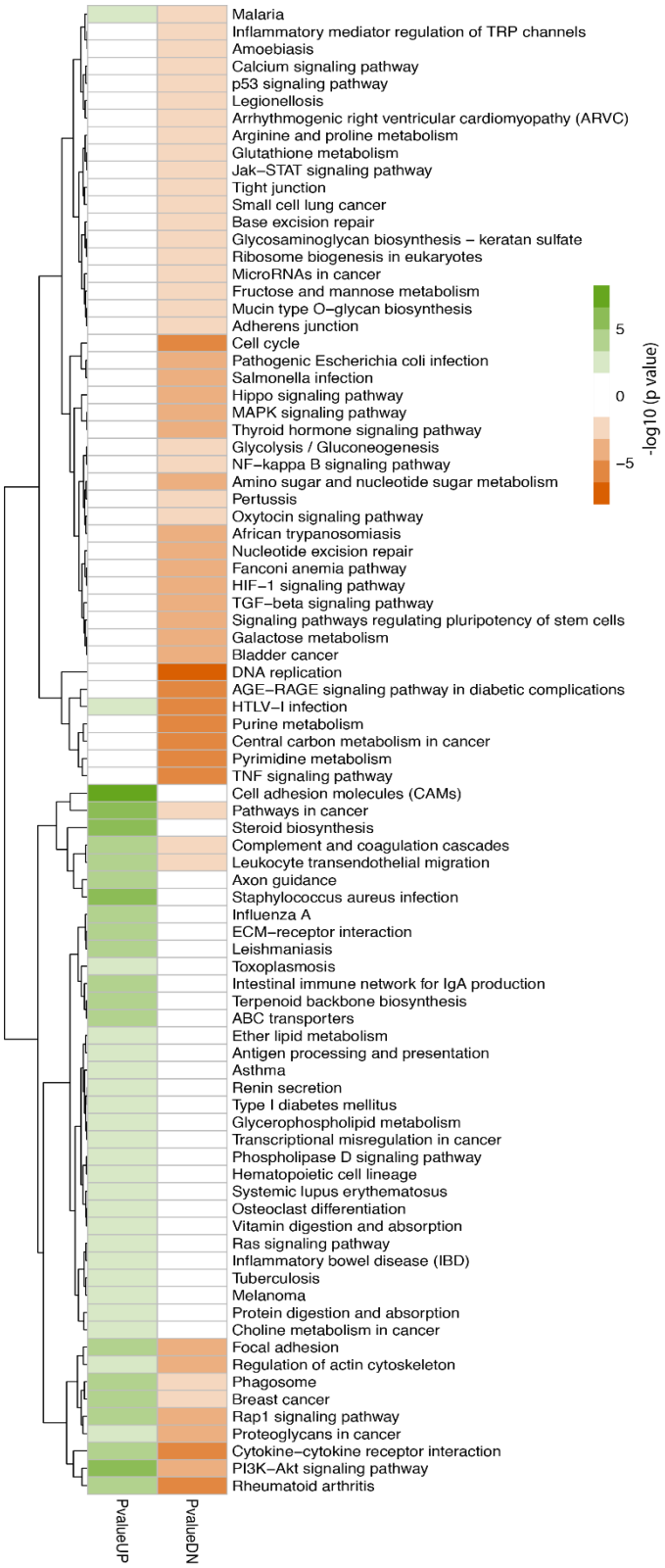


Figure 4.2.3d- *RNA Sequencing Analysis*. Kegg pathway enrichment for differentially up and down regulated genes. Colour represents strength of significant enrichment. Orange colour is for significant enrichment of down-regulated genes and green color is for significant enrichment of up-regulated genes within that particular pathway.

The transcipts per million (TPM) for each gene was calculated using R. Transcripts per million normalises the data for differences in sequence depths of samples and it is a way of analysing the differential gene expression profile of each sample. Transcripts per million (TPM) is used to estimate the relative molar concentration (rmc) of each mRNA species. The first step of TPM is to normalise for the gene length; longer genes will have more reads and this needs to be normalised for. The number of reads for each gene is divided by the gene length, this gives the reads per kilobase (RPK). The second step for TPM is to normalise for sequencing depth, some samples have higher sequencing depths than others, and thus will have more read counts for all the genes. The RPK for each sample is totalled and this total is then divided by a million to give a "per million" scaling factor. Then the read count for each gene which was normalised for gene length is divided by the new scaling factor of the sample it came from. The sum of the total normalised genes in each sample is the same when using TPM, this allows a comparison of the proportion of total reads that is mapped to each gene, between the samples (Wagner *et al*, 2012) (Figure 4.2.3e).

PC9 showed a clear seperation of knockdown samples and control samples. Samples that had PLCL-1 knocked down and treated with oxytocin clustered together and away from samples that did not have PLCL-1 knocked down but were treated with oxytocin. Samples that had PLCL-1 knockdown and were not treated with oxytocin clustered together and away from samples that did not have PLCL-1 knocked down and were not treated with oxytocin. Therefore the genes of interest that were chosen for RT-PCR validation were the gene that had the most influence on the

clustering seen in PC9 and these genes were identified by the coefficients from the loadings (Figure 4.2.3e) (Appendix 1, 2 and 3) . After the genes with the most influence on PC9 were identified, the genes that had very low read counts were exempted from further analysis.

Thereafter, the TPMs of the genes of interest were analysed to see if knocking down PLCL-1 had the same affect on the TPMs from all the patients. The TPMs were originally calculated as part of the transcriptomics analysis and corrected for read bias. If the TPMs showed that knocking down PLCL-1 had a variable effect on the expression of the gene, that gene was exempted from further analysis (Figure 4.2.3f).

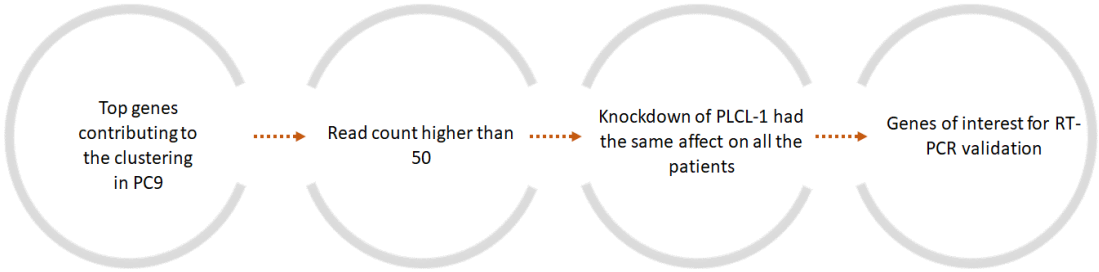
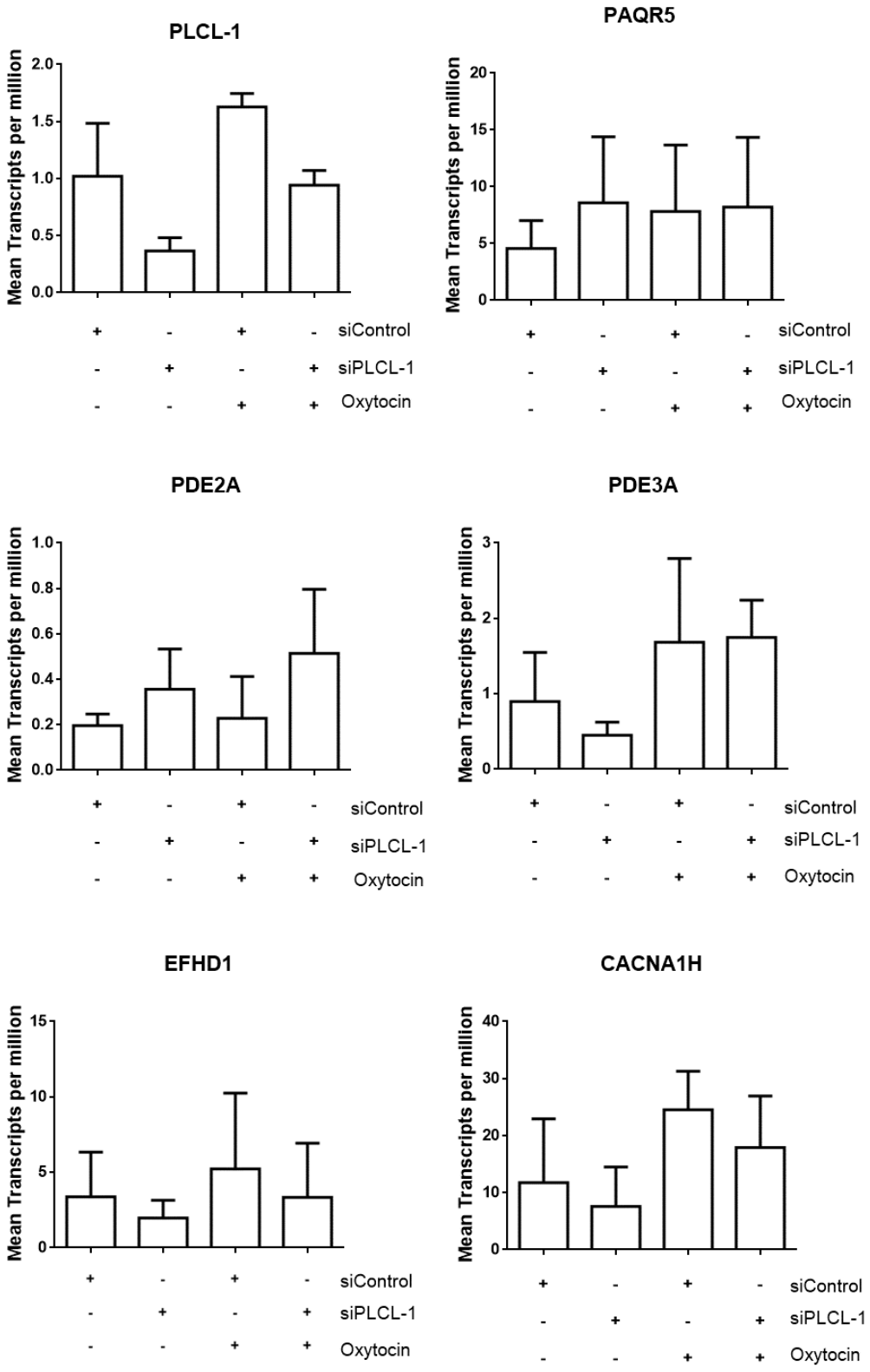


Figure 4.2.3e- ***Selection Criteria for the Genes of Interest***. The top contributing genes to the seperation in principal component 9 (PC9) were selected using the coefficients from loadings. Thereafter those genes that had a too low of a read count were exempted from further analysis. The transcript per million (TPM) of the genes were used to see if PLCL-1 knockdown had the same effect in all the patient, the genes that showed variable change in TPM count in the 6 different patients upon PLCL-1 knockdown, were exempted from further analysis. The genes that passed the selection criteria were analysed further using RT-PCR.


PLCL-1
Mean Transcripts per million
2.0
1.5
1.0
0.5
0.0
siControl
siPLCL-1
Oxytocin
PAQR5
Mean Transcripts per million
20
15
10
5
0
siControl
siPLCL-1
Oxytocin
PDE2A
Mean Transcripts per million
1.0
0.8
0.6
0.4
0.2
0.0
siControl
siPLCL-1
Oxytocin
PDE3A
Mean Transcripts per million
3
2
1
0
siControl
siPLCL-1
Oxytocin
EFHD1
Mean Transcripts per million
15
10
5
0
siControl
siPLCL-1
Oxytocin
CACNA1H
Mean Transcripts per million
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10
0
siControl
siPLCL-1
Oxytocin

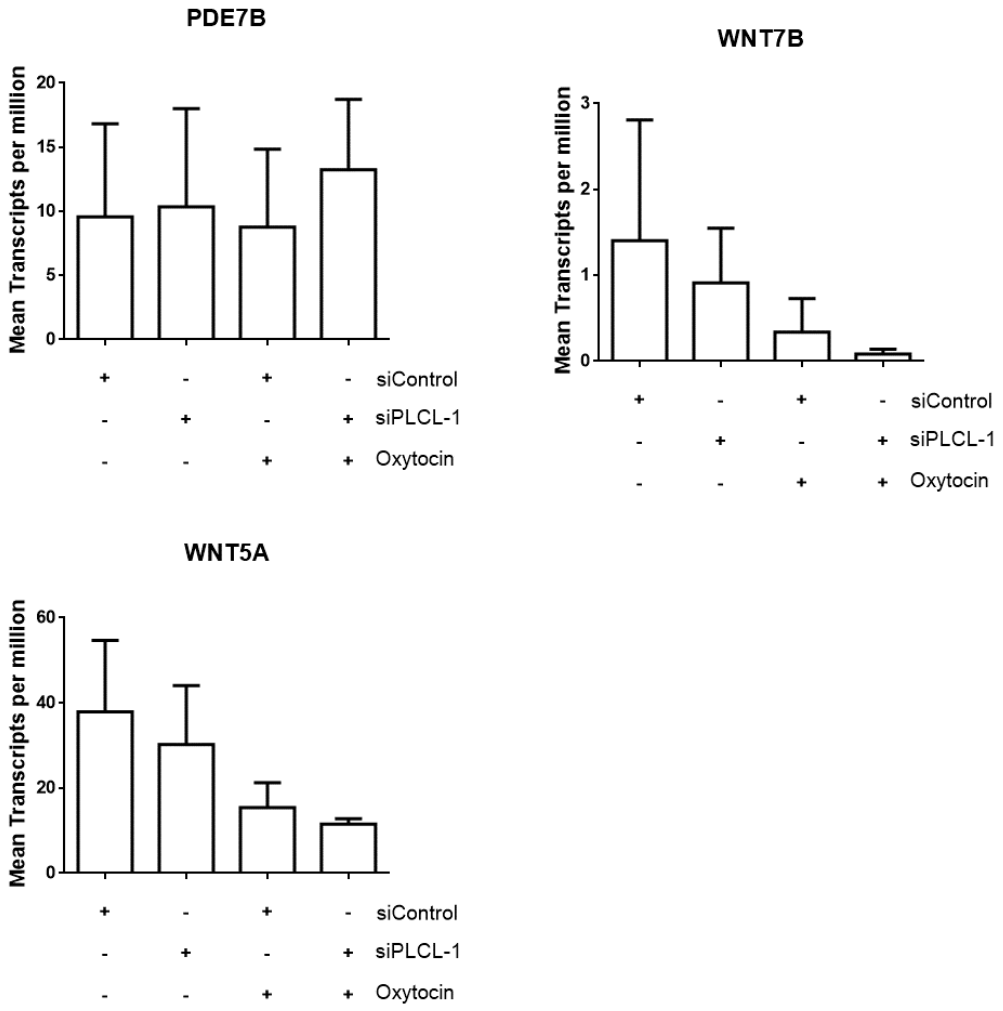


Figure 4.2.3f- ***Transcript Per Million*****.** Primary MSMCs were transfected with either siControl or siPLCL and with or without 10nM oxytocin.Total RNA from the cells was extracted for RNA sequencing, and the transcipt per million for each gene was calculated. The above genes represent 'genes of interest' from the RNA sequencing results.

There were 9 genes that met all the criteria for RT-PCR validation and they were: PLCL-1, WNT5A, EFHD1, PAQR5, CACNAH1, WNT7β, PDE2A, PDE3 and PDE7B. Treatment with siPLCL-1 showed a significant decrease in PLCL-1 mRNA. PLCL-1 knockdown also led to a decreasse in the mRNA of WNT5A, PAQR5 and EFHD1,

though this decrease was not significant. There was no changes in the mRNA of CACNAH1, after PLCL-1 knockdown. There was a increase in the mRNA of PDE2A AND PDE7B after PLCL-1 knockdown. The change seen in PDE2A was not significant but it was in PDE7. Treatment of primary myometrial cells with siPLCL-1 increased mRNA of PDE7B to by 1.8 folds, this increased even further by 2.4-fold when cells were also treated with 10nM oxytocin for 1.5 hours (Figure 4.2.3g).

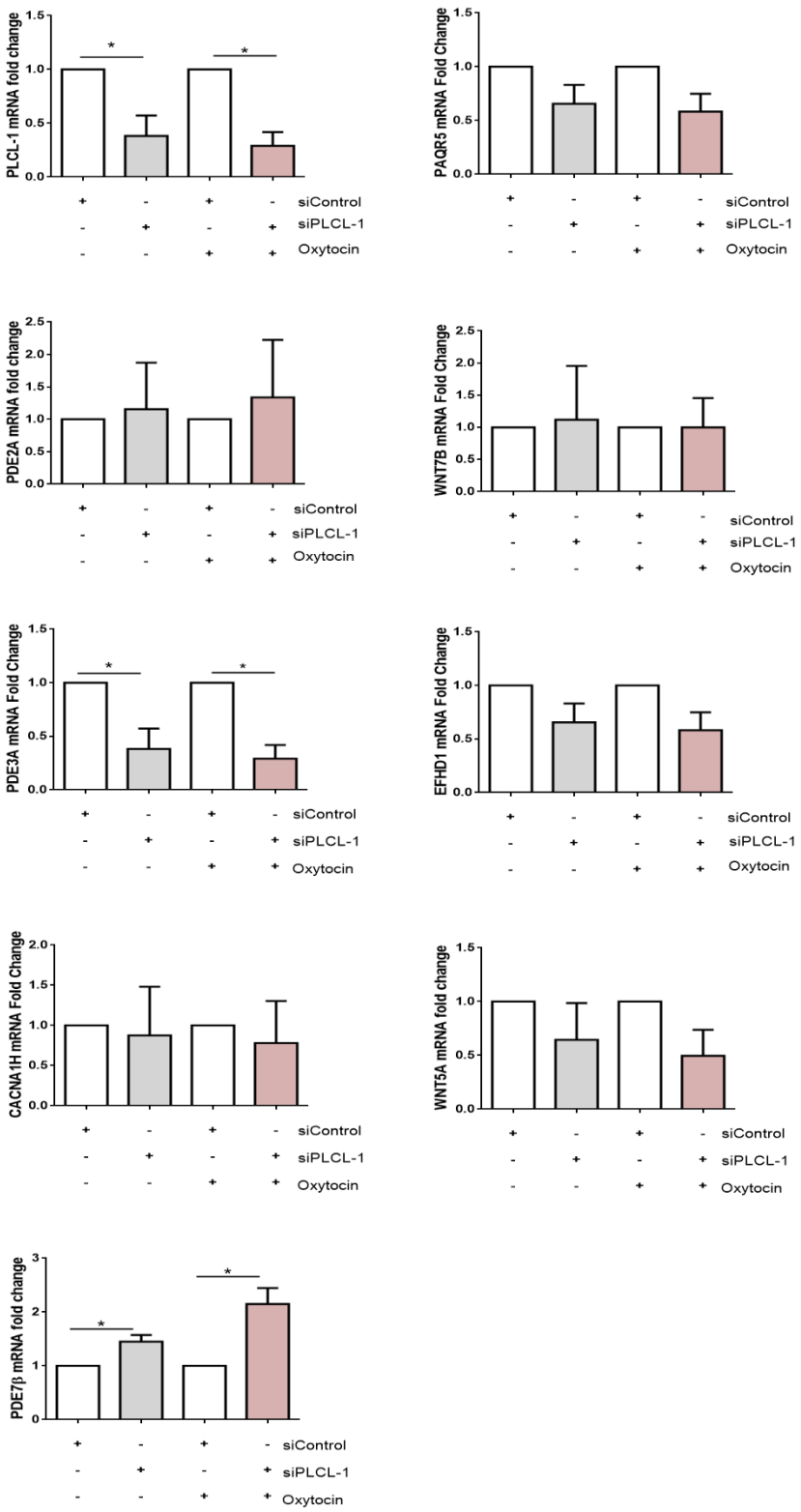


Figure 4.2.3g-*RT-PCR Validation of the Genes of Interest*. Primary MSMCs were transfected with either siControl or siPLCL for 48 hours and with (+) or without(-) 10nm oxytocin. Data was analysed using Student's t-test, $P \leq 0.05$ (*), n=3, error bars represent standard deviation.

4.2.4- Investigating the Effects PLCL-1 Knockdown has on cAMP Levels

The induction of PDE7B mRNA in response to PLCL-1 knockdown was replicated at the protein level(Figure 4.2.4a). Western blot analysis showed a 5 fold increase in protein production of PDE7B upon PLCL-1 knockdown. Western blotting was also conducted to investigate the effect of PLCL-1 knockdown on PDE3A expression, however there were no bands observed. PDE7B is part of the phosphodiesterase family of proteins. PDE7B degrades cAMP by catalysing the hydrolytic cleavage of the 3'-phosphodiester bond,resulting in the formation of the corresponding inactive 5'-monophosphate, leading to a reduction cellular levels of cAMP (Sasaki *et al*, 2000). As PLCL-1 knockdown increases expression PDE7B, the effect PLCL-1 knockdown has on cAMP production in Myla cells was examined using a cAMP assay.

A Homogeneous Time Resolved Fluorescence (HTRF) cAMP assay was used to examine whether PLCL-1 knockdown had any effect on cAMP production in myometrial cells. The endogenously produced cAMP competes for the binding for a cAMP recognising antibody with a fluorescently labelled cAMP, provided in the HRTF kit. The labelled cAMP is bound to a fluorescent acceptor (d2), a Europium cryptate fluorescent donor (Eu^{3+}) is added to the kit and this allows for there to be a resonance energy transfer between the fluorescent donor and acceptor, this produces a fluorescent signal (FRET signal). The more cellular cAMP there is present, the greater the loss of FRET signal. HRTF allows fluorescent emission to be measured at 2 different wavelengths; 620nm and 665nm. A standard curve is created using the ratio

between the 2 wavelengths with known concentrations of cAMP standards, using log concentrations. The standard curve is then used to extrapolate the concentration of cAMP in siControl and siPLCL-1 samples.

Knocking down PLCL-1 decreased cAMP concentrations in myometrial cells from 1000 pmol/L in siControl cells to 150 pmol/L when PLCL-1 was knocked down. Primary MSMC treated with forskolin saw a similar change in cAMP levels upon PLCL-1 knockdown, whereby cAMP concentration changed from 3,200 pmol/ml when treated with siControl to 500pmol/ml when the cells were treated with siPLCL-1. When a PDE7 specific inhibitor was added (BRL 50481) (Smith *et al*,2004), there was no significant difference in the concentration of cAMP in between siControl and siPLCL-1 cells, both had a concentration around 3000pmol/ml, when cells were treated with forskolin (Figure 4.2.4b).

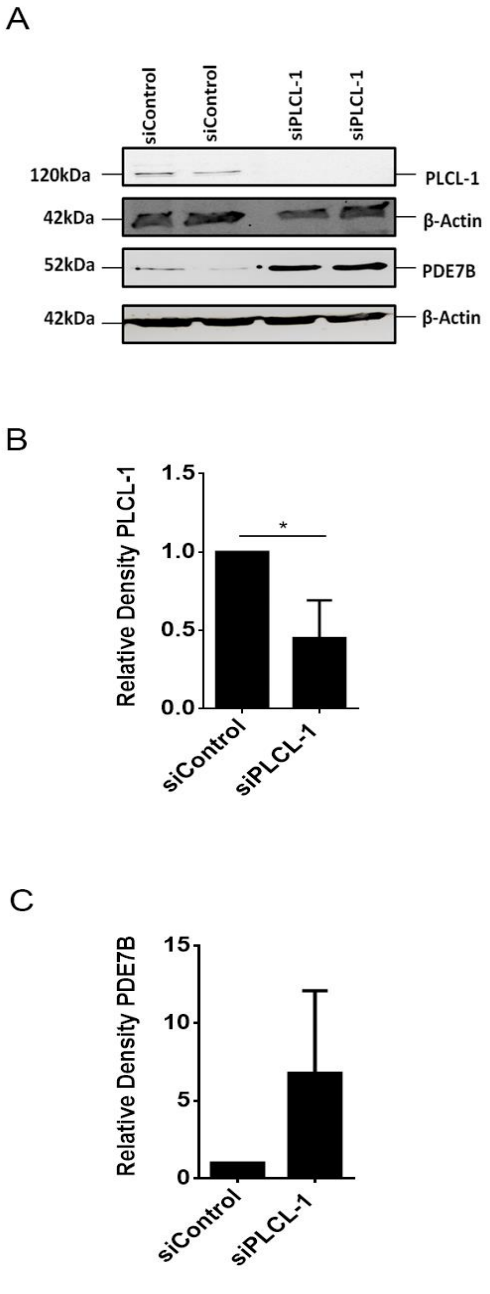


Figure 4.2.4a-*Effect of siPLCL-1 on Expression of PDE7B*. Primary MSMCs were transfected with siControl and siPLCL-1 . A) Western blot of transfected cell lysates. B&C) Western blot band density of siControl and siPLCL-1, relative to siControl. Data was analysed using Student's t-test, $P \leq 0.05$ (*), n=3, error bars represent standard deviation.

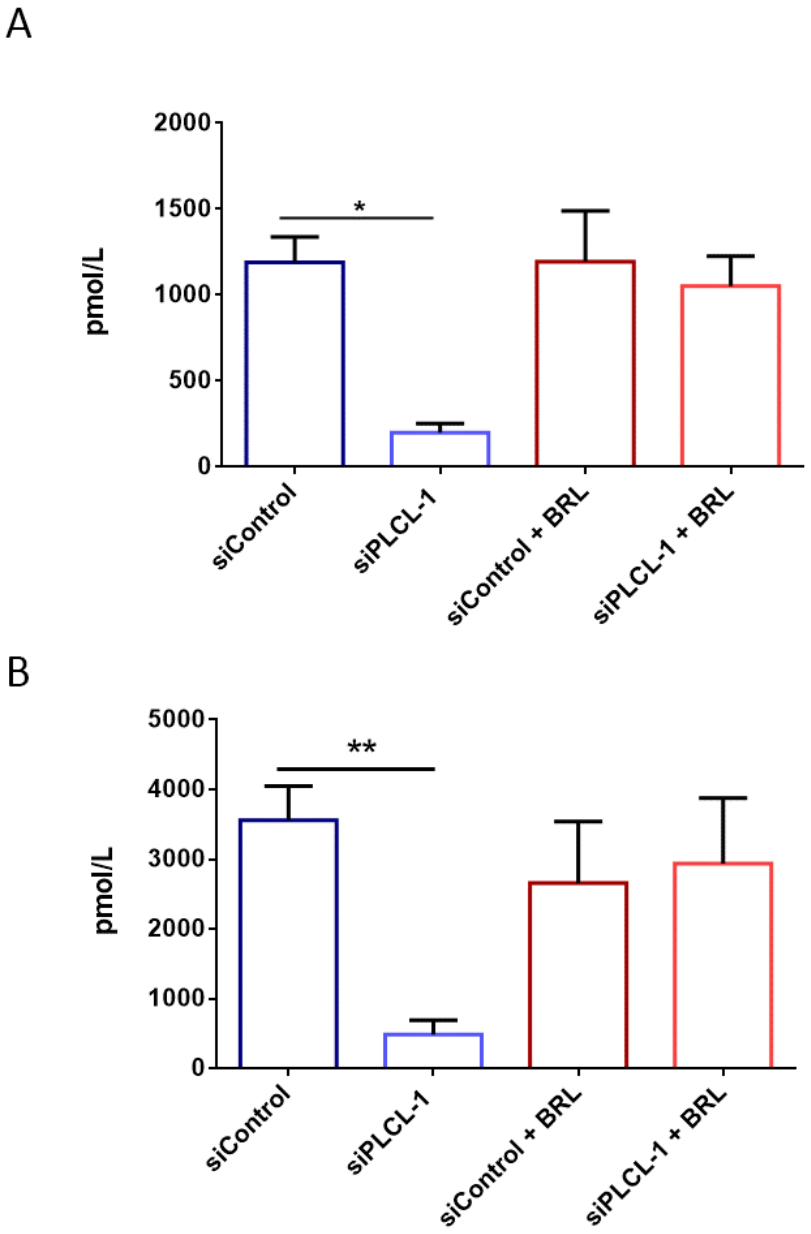


Figure 4.2.4b- ***Effect of siPLCL-1 and PDE7 Inhibitor on cAMP Production*****.** Primary Myometrial cells were transfected with siControl or siPLCL-1 for 48 hours; with or without PDE7 inhibitor, BRL 50481. Before cell harvesting, cells were treated without (a) or with (b) 100µM forskolin. Cells were then harvested for use in HRTF cAMP assay. Data was analysed using two-way ANOVA with Tukey's post hoc test, *P* ≤0.05 (*), n=3, error bars represent standard deviation.

4.3 Discussion

4.3.1- PLCL-1 Reduces Production of PDEs to Maintain Basal cAMP Levels

Calcium is a well characterised and important second messenger that can affect changes in gene expression. As PLCL-1 decreases $[Ca^{2+}]_i$ in MSMCs, the effects of PLCL-1 on the transcriptome was investigated. Analysis from RNA sequencing identified phosphodiesterases as 'genes of interest', in particular: PDE3A,PDE7B and PDE2A. RNA sequencing data showed that upon PLCL-1 knockdown there was an increase in the TPM of PDE7B and PDE2A, but a decrease in PDE3A. Validation of the RNA sequencing data using RT-PCR, showed that PLCL-1 was able to increase mRNA expression of PDE7B, but not PDE2A and PDE3A. Furthermore, western blot analysis illustrated that knockdown of PLCL-1 resulted in an increase in PDE7B protein detection.

There are eleven isoforms of PDEs and within each isoform there are different splice variants, leading to expression of more than 55 PDEs in mammalian cells. PDE's all have a conserved catalytic domain, located in the C-terminus that hydrolysis cAMP and cGMP into AMP and GMP respectively (Maurice *et al*, 2014) (Francis *et al*, 2011) (Keravis and Lugnier, 2014).

PDE7B mRNA is expressed the highest in the female reproductive organs particularly the ovary and the endometrium, in that order (Fagerberg *et al*, 2014). PDE7B has 64% structurally homologies with PDE7A, both PDE7A and PDE7B specifically target cAMP

and but has no affinity for cGMP (Sasaki *et al*, 2000). There are two splice variants of PDE7B: PDE7B2 and PDE7B3 (Sasaki *et al*, 2002). Cyclic AMP upregulates expression of PDE7B in a negative feedback loop and there are multiple phosphorylation sites present on PDE7B that are targeted for phosphorylation by PKA (Sasaki *et al*, 2004) (Sasaki *et al*, 2002).

Comparably to PDE7B, data from the RNA sequencing showed PLCL-1 knockdown increased the TPM count of PDE2A, however the effects on PDE2A were not validated using RT-PCR. Knockdown of PLCL-1 showed no significant change in PDE2A mRNA abundance

As PLCL-1 can modulate the expression of PDEs in MSMC, it was postulated that PLCL-1 may indirectly influence cAMP levels in MSMCs. PLCL-1 knockdown studies in MSMCs revealed a significant reduction in cAMP concentrations, suggesting that basal levels of cAMP are maintained by PLCL-1. The observed reduction in cAMP basal levels upon PLCL-1 knockdown was lost after PDE7 was inhibited. The combined data from the RNA-seq, western blotting and the cAMP assays, exemplifies a novel pathway whereby PLCL-1 indirectly keeps cAMP levels elevated by inhibiting phosphodiesterases from hydrolysing cAMP.

Cyclic AMP is a well known pro-quiescent molecule and it mediates its pro-quiescent effects through PKA. One mechanism by which cAMP inhibits contractions in the

myometrium is by inhibiting PLC in a PKA dependent manner. Certain isoforms of PLC have already been shown to be phosphorylated by PKA. For example, PLCβ_3 is phosphorylated by PKA at Ser^{1105}, which is situated in the $G_{\alpha q}$ subunit binding domain of PLCβ_3, this phosphorylation is thought to disrupt the binding of heterotrimeric G protein with PLC and consequently inhibit activation of PLC (Yue *et al,* 1998). Cyclic AMP inhibits increases in intracellular IP_3 concentration, induced by oxytocin. A PKA specific inhibitor reverses the cAMP attenuation of the oxytocin induced increase in IP_3 concentration in myometrial cells, this provides further evidence that cAMP inhibits myometrial PLC activity via PKA (Dodge and Sanborn, 1998).

PLCL-1 maintains the baseline levels of cAMP in the myometrium through its inhibition of phosphodiesterase. Knocking down PLCL-1 leads to an increase in PDE7B and a reduction in cAMP. In addition, PLCL-1 inhibits IP_3 mediated release in intracellular calcium stores (Chapter 3). As PLCL-1 binds directly to IP_3, it may be that PLCL-1 inhibits IP_3 by sequestering it. However, another mechanism by which PLCL-1 may inhibit IP_3 is by maintaining cAMP concentration in the myometrium, which subsequently inhibits PLC activity, which leads to a reduction the production of IP_3 from PIP2. The inhibitory effects of cAMP on PLC activity is lost in the rat myometrium during latter stages of gestation (Sanborn *et al*, 1995). PLCL-1 is downregulated during labour in both rodent (mice) and human myometrium, this may partly explain why cAMP is no longer able to inhibit PLC activity during late gestation.

Chapter 5

PLCL-1 Affects the Phosphorylation States of Kinases

5.1 Introduction

Post-translational modulation of kinases through phosphorylation affects a wide variety of cellular signalling pathways, causing numerous phenotypical changes. For example, in MSMC, a family of MAPK proteins (Figure 5.1.1) have been shown to be activated by stretch in rat myometrium (Louden *et al*, 2004). Stretch in rat myometrium activates the mitogen activated protein kinases: ERK1, JNK and p38α by increasing their phosphorylation levels. One study showed increased phosphorylation of ERK1, JNK and p38α 24 hours before parturition in the gravid horn of the pregnant rats and not in the empty horn, suggesting that this was due to pregnancy induced stretch (Oldenhof *et al*, 2002). This increased phosphorylation of MAPK led to the increased transcription of c-fos. Proteins c-fos and c-jun form a heterodimer, to make up the transcription factor AP-1. There are several CAPs which have AP-1 consensus binding sites for AP-1 in their promoter region including OTR and Cx43 (Mitchel and Lye, 2001).

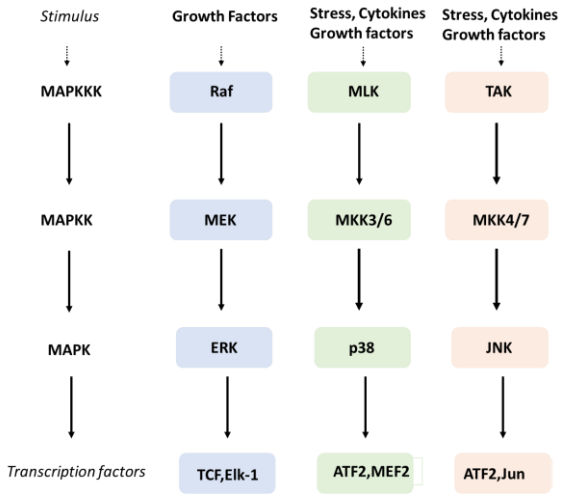


Figure 5.1.1 ***The MAPK Signalling Cascade***. ERK, p38 and JNK all are part of the MAPK family of proteins. A cellular stimulation event leads to a cascade of phosphorylation that activates the substrate kinase, eventually leading to the activation of transcription factors, and subsequent changes to the transcriptome.

PLCL-1 has been shown to bind the kinase AKT and affecting its phosphorylation levels in HESCs (Muter *et al*, 2016). PLCL-1 also binds to the phosphatases PP1 and PP2a, which have been shown to dephosphorylate MAPKs (Yu *et al*, 2004) (Alvarado-Kristensson and Andersson, 2005) (Kitatani *et al*, 2006). PLCL-1 decreases the activity of PP1 (Yoshimura *et al*, 2001). If PLCL-1 binds to and effects AKT phosphorylation levels in HESCs and modulates the activity of PP1, it might also do the same in MSMCs. PLCL-1 may be able to modulate transcriptional changes in MSMCs by regulating phosphorylation states of kinases, and this regulation may lead to a decrease in the expression of CAPS or increases in expression of pro-quiescent genes. A MAPK array assay was used to explore the ability of PLCL-1 to regulate the phosphorylation levels of kinases in MSMCs, this assay allows for observation of changes in phosphorylation levels of numerous MAPK in a time-efficient manner.

5.2 Results

5.2.1- The Effect of PLCL-1 Knockdown on Phosphorylation of Mitogen Activated Protein Kinases (MAPK)

Primary myometrial cells were transfected with either siControl or siPLCL-1 for 48 hours and thereafter harvested for use in a Phospho-MAPK antibody array kit/dot blot (Figure 5.2.1). In the dot blot, Phospho- specific antibodies are dotted onto a nitrocellulose membrane and phosphorylation levels are detected using chemiluminescence. The dot blot has 6 reference spots as positive controls, to demonstrate that the Streptavidin-HRP is working and to align the membrane in the

correct orientation. The pixel density of each phospho- kinase was analysed using imageJ. Pixel density from each dot is calculated as a percentage of the density from the positive control reference spots, and this is plotted in the y axis.

AKT/PKB is a serine/threonine specific protein kinase, there are three known isoforms of AKT: AKT1, AKT2 and AKT3. The phospho-MAPK dot blot has antibodies specific for the phospho-AKT1, AKT2, AKT3 and total phospho-AKT (AKT-PAN). In siControl samples the mean phosphorylation levels as a percentage of reference spot density in AKT1, AKT2, AKT3 and AKT-PAN were 5.98 32.36, 17.67 and 19.21 (± SD 0.94, 2.11, 1.44, and 1.15) respectively. There was a significant reduction in the mean phosphorylation of AKT1 and AKT3 after PLCL-1 knockdown to 1.49% (± SD 1.23) and 1.69% (± SD 1.36), respectively. However, there was no significant reduction in phosphorylation levels of AKT2 and AKT-PAN (Figure 5.2.2.).

There was a no significant change in cAMP response element binding protein (CREB) phosphorylation levels upon PLCL-1 knockdown, Similarly to CREB, upon PLCL-1 knockdown, there was no significant change in mean phosphorylation levels of ERK1 and ERK 2 (Figure 5.2.2).

There was no significant change in the mean phosphorylation levels of the glycogen synthase kinase isoforms: GSK-3 a/ß and GSK. Mean phosphorylation levels of heat

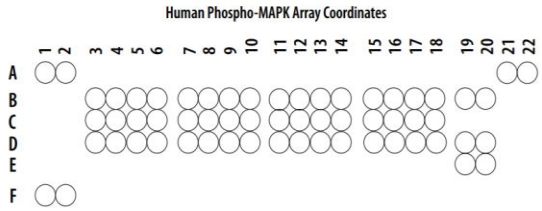
shock protein 27 (HSP27) decreased from 62.42 % (± SD 3.31) to 29.61% (± SD 8.71), when PLCL-1 was knocked down (Figure 5.2.3).

There were no significant changes in the phosphorylation levels of the c-Jun terminal kinase isoforms: JNK1, JNK2 or JNK3 (Figure 5.2.3). Kinases MKK6 and MSK2, mean phosphorylation levels decreased from 16.73% (± SD 0.79) and 29.57% (± SD 1.62) to 5.04% (± SD 1.44) and 6.49% (± SD 2.58), respectively, upon treatment with siPLCL-1(Figure 5.2.4). Kinase MKK3, saw no significant change in the phosphorylation state in siPLCL-1 lysates in comparison to siControl lysates.

Two of the P38 MAPK isoforms saw a marked decrease in phosphorylation states in response to PLCL-1 knockdown. Isoforms P38α and P38β saw a significant decrease in phosphorylation from 29.65% (± SD 6.65) and 25.68% (± SD 1.61) to 3.00% (± SD 2.52) and 2.96% (± SD 1.39), respectively (Figure 5.2.4). However there was no significant change in the phosphorylation levels of isoforms p38δ and p38γ.

There was no change in the phosphorylation state of p53, however p70 s6 kinase saw an increase from 5.22% (± SD 1.03) to 25.96% (± SD 3.84). Kinases RSK1, RSK2 or TOR saw no significant change in phosphorylation levels, when PLCL-1 was knocked down (Figure 5.2.5).

A



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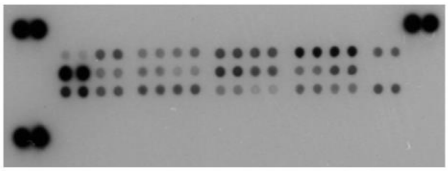

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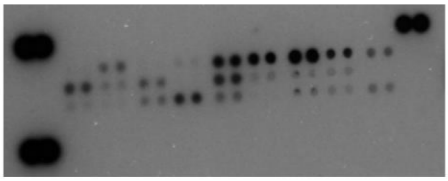

D

AKT1 S473	B3,B4
AKT2 S474	B5,B6
AKT3 S472	B7,B8
AKT PAN S473 S474 S472	B9,B10
CREB S133	B11,B12
ERK1 T202/Y204	B13,B14
ERK2 T185/Y187	B15,B16
GSK 3α/β S21/S9	B17,B18
GSK 3β S9	B19,B20
HSP27 S78/S82	C3,C4
JNK1 T183/Y185	C5,C6
JNK2 T183/Y185	C7,C8
JNK3 T221/Y223	C9,C10
JNK PAN T183/Y185,T221/Y223	C11,C12
MKK3 S218/T222	C13,C14
MKK6 S207/T211	C15,C16
MSK2	C17,C18
P38α T180/Y182	D3,D4
P38β T180/Y182	D5,D6
P38?T180/Y182	D7,D8
P38γ T183/Y185	D9,D10
P53 S46	D11,D12
P70 S6 Kinase T421/S424	D13,D14
RKS1 380	D15,D16
RSK2 S386	D17,D18
TOR S2488	D19,D20
PBS control	E19,E20
Reference spot	A1,A2,A21,A22, F1,F2

Figure 5.2.1 -*Phospho-MAPK Assay*. A) Schematic representation of array coordinates. B) Example blot from siControl lysate from primary MSMCs. C) Example blot from siPLCL-1 lysate from primary MSMCs. D) Table showing the corresponding kinases antibodies to the array coordinates.

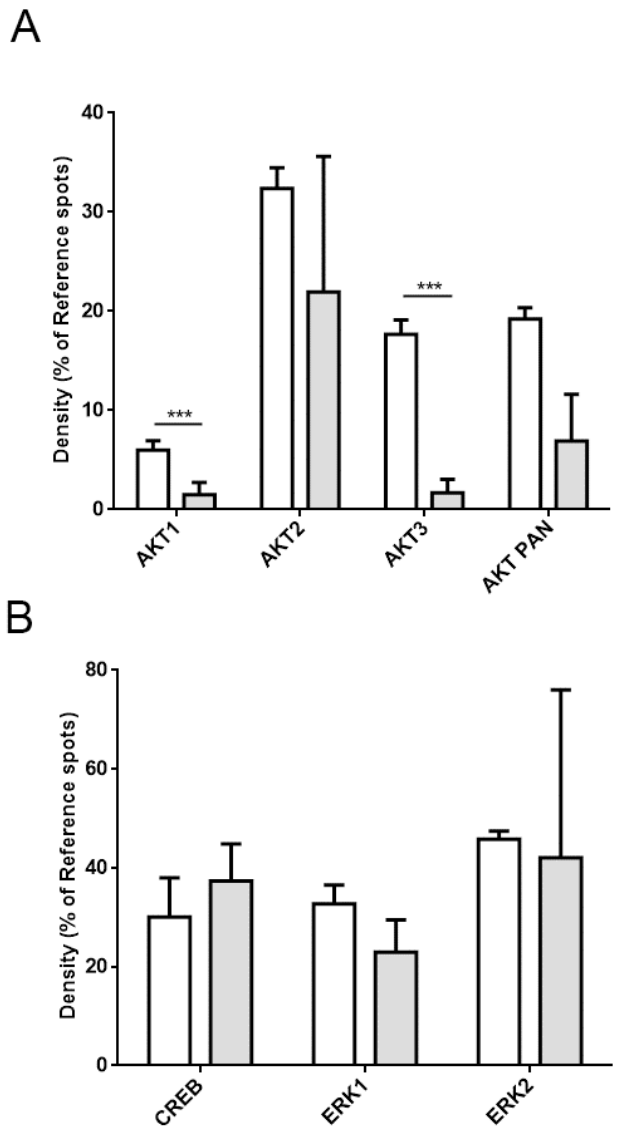

Figure 5.2.2- ***Effect of siPLCL-1 on Phosphorylation of Kinases*.** Primary myometrial cells were treated with siControl (white bars) or siPLCL-1 (grey bars) for 48 hours. Cells were harvested for use in phospho- MAPK dot blot. Phosphorylated kinases were detected using chemiluminescence, and the density analysed as a percentage of reference spots. Data was analysed using Student's t-test, $P \leq 0.05$ (*), n=3, error bars represent standard deviation.

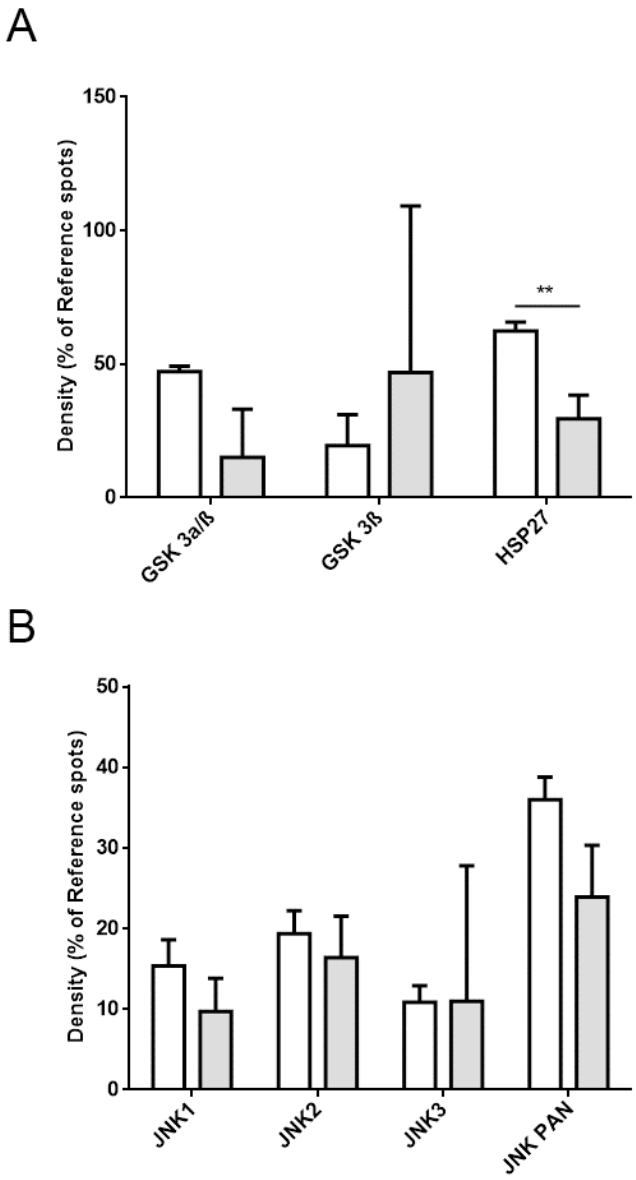

Figure 5.2.3- ***Effect of siPLCL-1 on Phosphorylation of Kinases*****.** Primary myometrial cells were treated with siControl (white bars) or siPLCL-1 (grey bars) for 48 hours. Cells were harvested for use in phospho- MAPK dot blot. Phosphorylated kinases were detected using chemiluminescence, and the density analysed as a percentage of reference spots. Data was analysed using Student's t-test, $P \leq 0.05$ (*), n=3, error bars represent standard deviation.

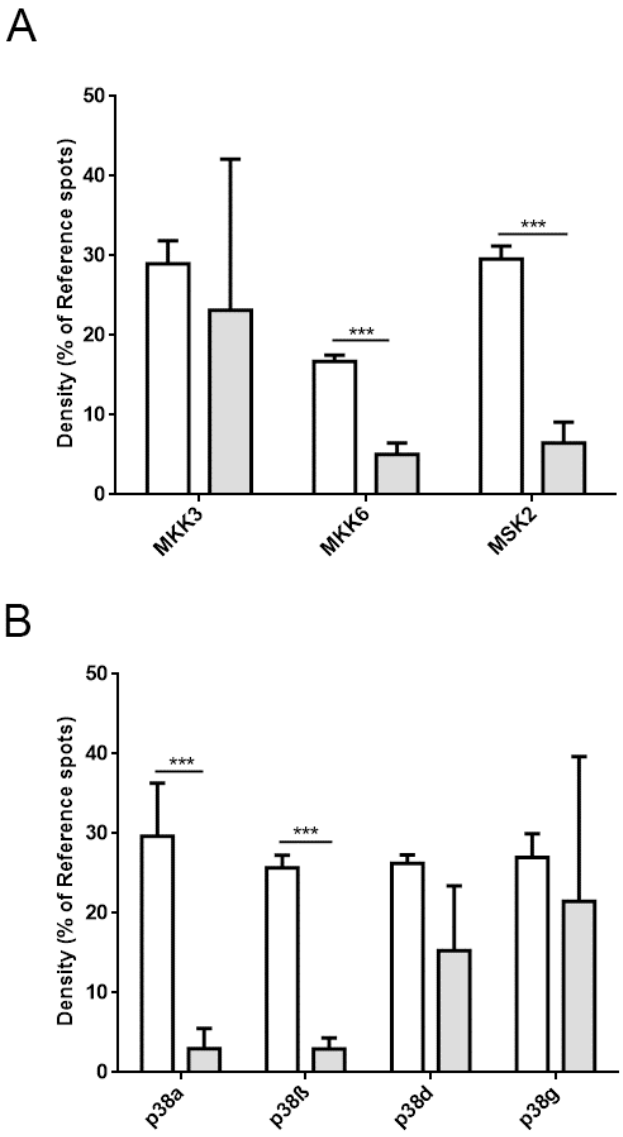

Figure 5.2.4- ***Effect of siPLCL-1 on Phosphorylation of Kinases*****.** Primary myometrial cells were treated with siControl (white bars) or siPLCL-1 (grey bars) for 48 hours. Cells were harvested for use in phospho- MAPK dot blot. Phosphorylated kinases were detected using chemiluminescence, and the density analysed as a percentage of reference spots. Data was analysed using Student's t-test, $P \leq 0.05$ (*), n=3, error bars represent standard deviation.

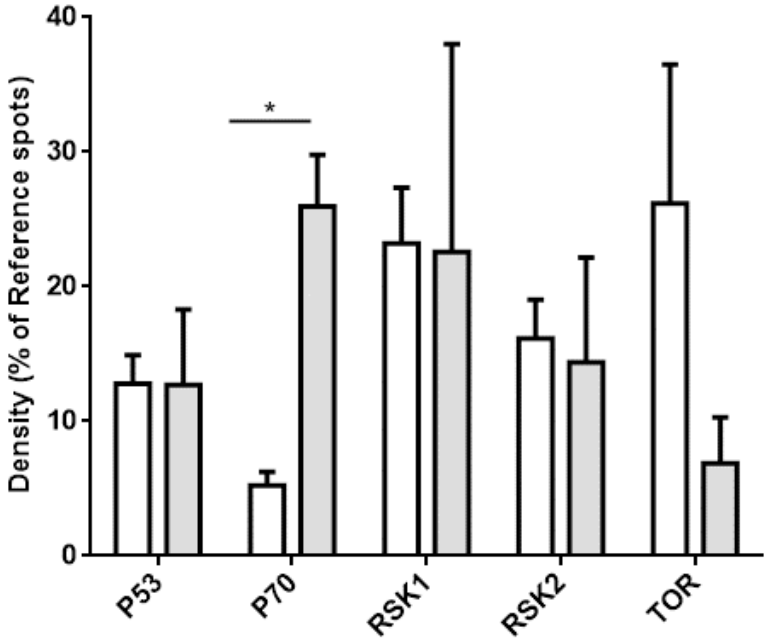

Figure 5.2.5- ***Effect of siPLCL-1 on Phosphorylation of Kinases*****.** Primary myometrial cells were treated with siControl (white bars) or siPLCL-1 (grey bars) for 48 hours. Cells were harvested for use in phospho- MAPK dot blot. Phosphorylated kinases were detected using chemiluminescence, and the density analysed as a percentage of reference spots. Data was analysed using Student's t-test, $P \leq 0.05$ (*), n=3, error bars represent standard deviation.

5.3 Discussion

PLCL-1 knockdown significantly decreases phosphorylation of Akt1, Akt2 and Akt3 in MSMCs, the same effect was shown in HESCs (Muter *et al*, 2016). In addition to Akt, a loss of PLCL-1 also leads to a reduction in phosphorylation in other proteins such as: GABA(A) receptor and SNAP-25 (Terunuma *et al*, 2004) (Gao *et al*, 2012). Therefore, one of the functions of endogenous PLCL-1 is to maintain phosphorylation states of protein. PLCL-1 can do this using two methods: 1) increase kinase activity 2) reduce phosphatase activity. Previous studies have shown that PLCL-1 is able to reduce activity of the phosphatase PP1 (Yoshimura *et al*, 2001). Expression of PLCL-1 reduces PP1 mediated dephosphorylation of SNAP-25 (Gao *et al*, 2012). PLCL-1 may maintain phosphorylation of Akt by reducing the activity of PP1 (Figure 5.3.1). PP1 regulates the ability of AKT to modulate gene expression by de-phosphorylating Akt at Thr-450. (Xiao *et al*, 2010) (Thayyullathi *et al,* 2011). However, there is more evidence showing that AKT is a substrate of PP2a (Andjelković *et al*, 1996) (Meier *et al*, 1998) (Di Maira *et al*, 2009) (Kuo *et al*, 2008). PLCL-1 binds both PP1 and PP2a, it may be that PLCL-1 regulates their activity by sequestering them away from their substrates. As previously mentioned phosphorylation of PLCL-1 modulates the binding of PLCL-1 to PP1 and PP2a. Phosphorylation of PLCL-1 causes release of PP1 and increased binding of PP2a.

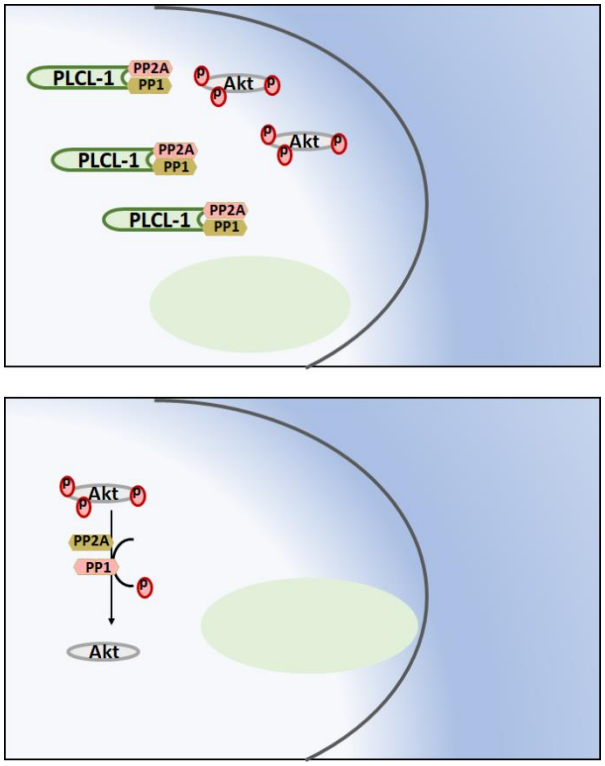

Figure 5.3.1-*PLCL-1 Regulates Phosphorylation of Akt*. When PLCL-1 levels are high; PLCL-1 can inhibit PP1 and PP2a, thus Akt is kept in a phosphorylated state. The loss of PLCL-1, frees PP1 and PP2a to dephosphorylate Akt, thus Akt is inhibited.

Akt is activated via phosphorylation. The first step in Akt activation is by JNK1 and JNK2 which phosphorylates Akt at Thr-450 (Shao *et al*, 2006) (Shaw and Kirshenbaum, 2006). Thereafter PI3K activation leads to it converting PIP_2 into PIP_3, which recruits PDK1 and Akt to the plasma membrane, allowing PDK1 to phosphorylate at Thr-308 (Stephens *et al* 1998) (Alessi *et al*, 1996). Phosphorylation of Akt at Thr-308 causes a conformational change, revealing a hydrophobic motif in its C-terminus, this conformational change allows Akt to auto-phosphorylate or be phosphorylated by other kinases at Ser-473, causing Akt to be fully activated so that it may phosphorylate its target proteins (Behn-Krappa

and Newton, 1999) (Sarbassov *et al*, 2005).PLCL-1 keeping Akt in a phosphorylated state, will allow Akt to be continuously active.

AKT is a serine/threonine kinase and is involved in numerous signalling pathways; PLCL-1 regulating AKT phosphorylation, leads to PLCL-1 indirectly regulating substrates of AKT. An interesting AKT pathway in MSMCs is the PI3K/AKT/mTOR pathway. mTOR is a serine/threonine kinase and it exists in two complexes mTORC1 and mTORC2, mTORC1 is involved in cell growth while mTORC2 is involved in sell survival (Saxton and Sabatini,2017). Akt activates mTORC1 by phosphorylating the inhibitory subunit of mTORC1 called PRAS40, this causes it to dissociate from mTORC1, (Sancak *et al*, 2007) (Vander Haar *et al*, 2007).

Once activated, mTORC1 has numerous downstream targets, such as Cx43. mTORC1 inhibits expression of Cx43, and subsequent gap junction formation (Smyth and Shaw,2014) (Shen et al, 2016). Nadeem et al showed that PRA expressing myometrial cells expressed Cx43, which formed gap junctions. Whilst PRB expressing myometrial cells had reduced expression of Cx43 and gap junctions, and this reduction was mediated by mTORC1 signalling, as inhibition of mTORC1, restored expression of Cx43 expression (Nadeem et al, 2016). Interestingly mTORC1 expression is reduced in myometrium of women IL compared to NIL (Foster et al, 2014). mTORC1 expression was also decreased in placental samples from women IL compared to NIL (Lager et al, 2014). Additionally, expression of mTORC1 decreased in rat myometrium IL samples compared to NIL samples (Jaffer *et al*, 2009).

Reduction of mTORC1 expression in myometrium during late gestation and labour, will stop the inhibition of mTORC1 on Cx43 expression to allow for gap junction formation and synchronised MSMCs contraction. A loss of PLCL-1 during labour, may cause reduction in AKT signalling and no activation of mTORC1, which then removes the inhibition of mTORC1 on Cx43 expression (Figure 5.3.2). It would be expected that Cx43 to be one of the differentially expressed genes identified from the RNA sequencing data, however Cx43 was not one of the top contributing genes contributing to the gene cluster seen in PC9. Nevertheless, the modulatory action of PLCL-1 on AKT, could lead to PLCL-1 affecting downstream targets of AKT.

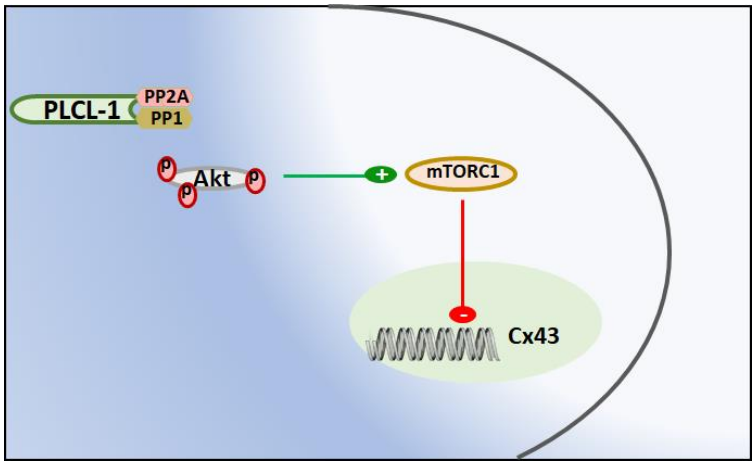

Figure 5.3.2- ***PLCL-1 may Indirectly Affect Downstream Targets of AKT.*** PLCL-1 inhibits phosphatases (PP1 and PP2a), thus Akt stays phosphorylated and active. Akt activates mTORC1, which inhibits expression of Cx43.

HSP27 and Actin Filament Stabilisation

Monomeric globular actin (G-actin) undergo polymerisation into a filamentous actin (F-actin). Myosin head binds to F-actin during contraction, therefore actin polymerisation is crucial for contraction and tension development in smooth muscle cells (Mehta and Gunst, 1999). There is evidence showing that isoforms of heat shock protein (HSP) are involved in actin filament stabilisation (Gerthoffer and Gunst, 2001). HSPs can form large homo or hetero-oligomeric complexes. Kinases regulate HSP activity, phosphorylated HSP leads to a dissociation of the oligomeric complexes, permitting smaller phosphorylated HSPs dimers or oligomers to interact with their targets. The functional aspects of HSPs are mainly controlled by phosphorylation, which stimulates a transformation in the tertiary structure HSPs, thus changing their affinity to form homo- or hetero-oligomers or to interact with actin filaments (Gusev *et al*, 2002).

Isoforms of HSP have been associated with modulating both contraction and relaxation of MSMCs. Phosphorylation of HSP20 at serine-16 by PKA has been shown to bind to α-smooth muscle actin and promote relaxation of the myometrium (Tyson *et al*, 2008). In contrast, HSP27 has been implicated in promoting myometrial contractions. There is increased phosphorylation of HSP27 at serine-15 during labour. Phosphorylation of HSP27 at serine-15 has been shown to stabilise F-actin filaments. Therefore, phosphorylation of HSP27 may promote uterine contractility by stabilising actin filament polymerisation (MacIntyre *et al*, 2008).

The MAPK array assay (Figure 5.4.3) showed a decrease in HSP27 phosphorylation in MSMCs upon PLCL-1 knockdown. Therefore, a reduction in PLCL-1 levels may lead to a decrease in uterine contractility due to the reduction in HSP27 phosphorylation and the subsequent loss in actin filament stabilisation. If PLCL-1 functions as a pro-quiescent protein it would be expected that a loss in PLCL-1 would lead to an increase in HSP27 phosphorylation, however the MAPK array assay showed a contrasting result.

Chapter 6

PLCL-1 is a Progesterone Responsive Gene.

6.1 Introduction

The classical pathway of PR transcriptional control is thought to follow distinct stages. Unliganded PR is in the cytoplasm and bound by regulatory heat shock proteins (Kost et al, 1989) (Onate et al, 1991). Upon P4 binding the heat shock proteins dissociate, PR forms either homodimers or heterodimers and translocate to the nucleus (Smith, 1993) (DeMarzo *et al*, 1991) (Guiochon-Mantel *et al*,1989). Within the nucleus, PR localises to palindromic DNA binding sites which have consensus PRE-regions (GnACAnnnTGTnC) in the promoter of PR responsive genes (Chalepakis *et al*, 1998) (Mulvhill *et al*, 1982) (Lieberman *et al*, 1993) (Yin *et al,* 2012). PR recruits transcriptional co-regulators to modulate transcription (Onate *et al*, 1995). However, this classical pathway is challenged by data that shows monomer PRs can activate transcription (Connaghan-Jones *et al*, 2008) (Jacobsen *et al*, 2009), unliganded PR can activate transcription and the PRE-sequence is still not precisely well-defined (Bamberger *et al*, 1996). Studies on PR transcription have been conducted using many different methods including: luciferase assay with promoters containing PREs, deletion of promoters, EMSA (electrophoretic mobility shift assay), DNA foot printing or methylation. More recently ChIP (chromatin immunoprecipitation) assays have been used to search for PR binding sites, and this is used in combination with DNA sequencing (ChIP-seq) or microarrays (ChIP-on-ChIP).

In the pregnant myometrium PR keeps the myometrium in a non-contractile state by upregulating pro-quiescent genes and downregulating CAPs. One method of

progesterone 'functional withdrawal' during term is a loss in PRB mediated transcriptional modulation, causing an increase in CAPs expression and decrease in pro-quiescent genes . Previous studies on NIL vs IL myometrium transcriptome studies show a significant reduction in PLCL-1 expression, suggesting that PLCL-1 is an important pro-quiescent gene, which indicates that with its loss might be needed for parturition. If PLCL-1 is a pro-quiescent gene than it might be a P4 responsive gene.

It is hypothesised that PLCL-1 acts a pro-quiescent protein by functioning as an effector protein for progesterone. PLCL-1 operates as an effector of progesterone by inhibiting the release of intracellular calcium, and thus inhibiting contractions. In humans, progesterone concentrations do not decrease before the onset of labour. In contrast, PLCL-1 levels do decrease before labour, thus the functional withdrawal of progesterone might be through the loss of PLCL-1. To test this hypothesis, it must first be established that the expression of PLCL-1 is modulated by progesterone, this was done by doing simple qRT-PCR and western blotting experiments.

If PLCL-1 proved to be a P4 responsive gene I wanted to take a closer look at the specific site of PR binding to the PLCL-1 promoter by utilising ChIP-qPCR.

If PLCL-1 is a progesterone effector protein, PLCL-1 can then be used as biomarker that identifies women who are more at risk for premature labour. It would be expected that women who are close to term or preterm labour would express less

PLCL-1 than women not in labour. A good clinical test would identify women at risk for preterm labour as early as possible. All the previous studies show a loss in PLCL-1 during labour in the myometrium. However, it is not feasible to take myometrial biopsies from women who are not undergoing caesareans. A better method to check if there is a reduction in PLCL-1 levels would be to take a blood sample. A blood sample can also be taken at any time during the pregnancy, and can identify high risk women early, allowing for earlier clinical interventions. There is no study indicating that PLCL-1 is expressed by white blood cells, however studies have shown that PLCL-1 is not exclusively expressed in the uterus but is also expressed by different tissues in the body. It must first be established that PLCL-1 is expressed by leukocytes and can be quantified from blood samples, therefore the concentration of PLCL-1 in whole blood was measured using qPCR.

6.2 Results

6.2.1- Effect of Medroxyprogesterone Acetate (MPA), P4 and cAMP Treatment on PLCL-1 Expression

To determine the effects of MPA and cAMP on the transcription and translation of PLCL-1, primary myometrium cell lines were treated with MPA and cAMP for 96 hours and then harvested for western blot and RT-PCR analysis. MPA is a derivative of 17α-hydroxyprogesterone, and is a potent agonist of the progesterone receptor, cAMP and progesterone work synergistically to activate progesterone dependent pathways and together amplify the effects of MPA (Gellersen and Brosens, 2003). Treating cells with cAMP and MPA, increased PLCL-1 transcription by 2.2-fold (SD ± 0.28) and 2.5-fold (SD ± 0.56) respectively. Treating the cells with cAMP combined with MPA also increased PLCL-1 transcription, to 2.5-fold (SD ± 0.30). Treating the cells with cAMP, MPA and cAMP/MPA increased PLCL-1 protein expression by a 1.40, 1.87 and 2.42 folds (SD ± 0.05,0.34, 0.25), respectively (Figure 6.2.1a).

The cells were treated with progesterone (P4) and the effect P4 had on PLCL-1 production in primary myometrial cells was examined. P4 treatment alone, increases PLCL-1 mRNA by 2.2-fold (SD ± 0.11) in comparison to control. Treatment of P4 in combination with cAMP, increased PLCL-1 mRNA by 2.8-fold (SD ± 1.65). The largest increase in PLCL-1 mRNA production was seen when cells were treated with P4 and MPA together. P4 treatment of primary myometrial cells increased PLCL-1 protein levels by 4-fold (SD ± 0.51) compared to control. Treatment of cells with P4 and cAMP

or P4 and MPA increased protein production of PLCL-1 by 6-fold (SD ± 1.18) (Figure 6.2.1b). MPA has a higher binding affinity for PR compared to P4, however it has less binding specificity than P4. MPA binds to both androgen receptor and glucocorticoid receptor, which P4 does not and has a much higher binding affinity for mineralocorticoid receptor compared to P4 (Schindler *et al*, 2003). The reduction in binding specificity of MPA compared to P4, may explain why P4 is a stronger inducer of PLCL-1 expression.

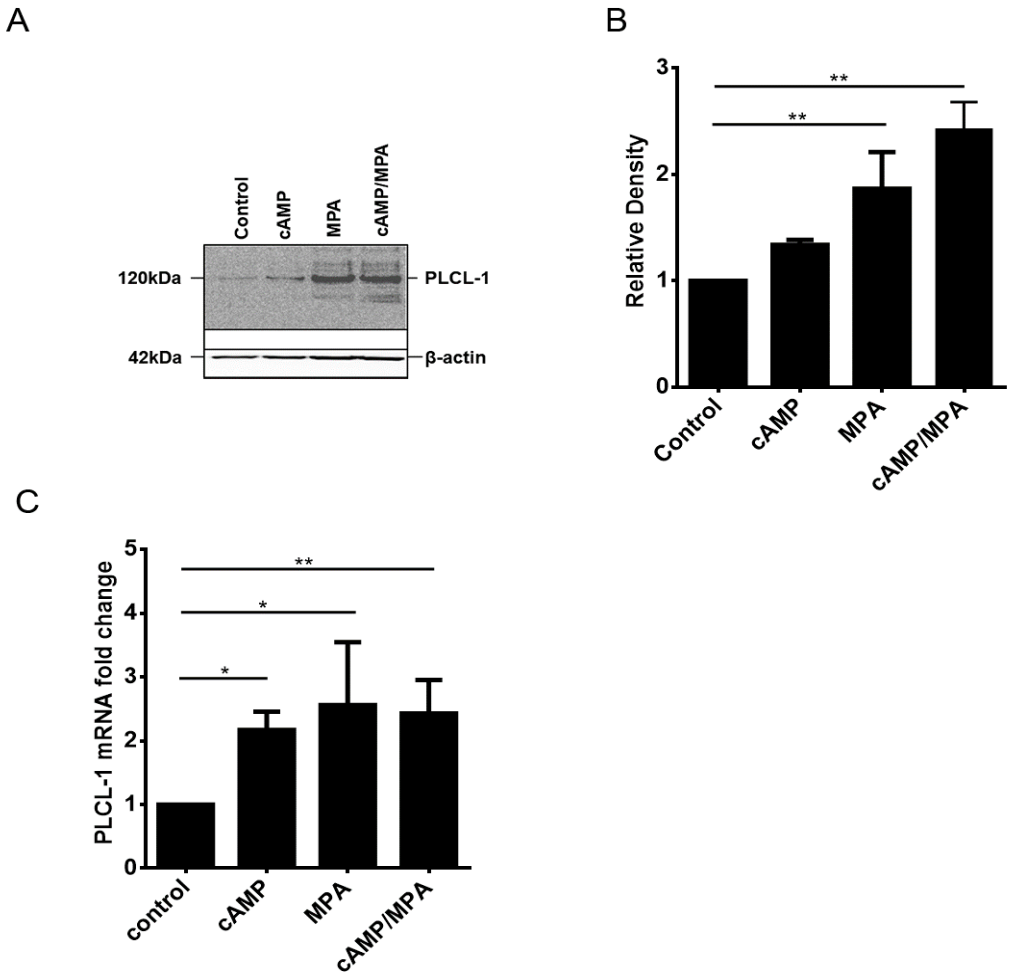

Figure 6.2.1a-*Effect of cAMP and MPA on PLCL-1 Expression*. Primary myometrium cells were serum starved for 24 hours and treated with either cAMP, MPA or cAMP and MPA together. A) Cells were harvested after 96 hours of treatment and cell lysates were analysed for PLCL-1 protein expression by Western blot. B) Band intensity from western blot was calculated and treated samples compared relative to control. C) mRNA fold change of PLCL-1 was analysed using RT-PCR. Data was analysed using one-way ANOVA with Dunnett's post hoc test. MPA and cAMP/MPA treated cells were compared to control, n=3, $P \leq 0.05$ (*).

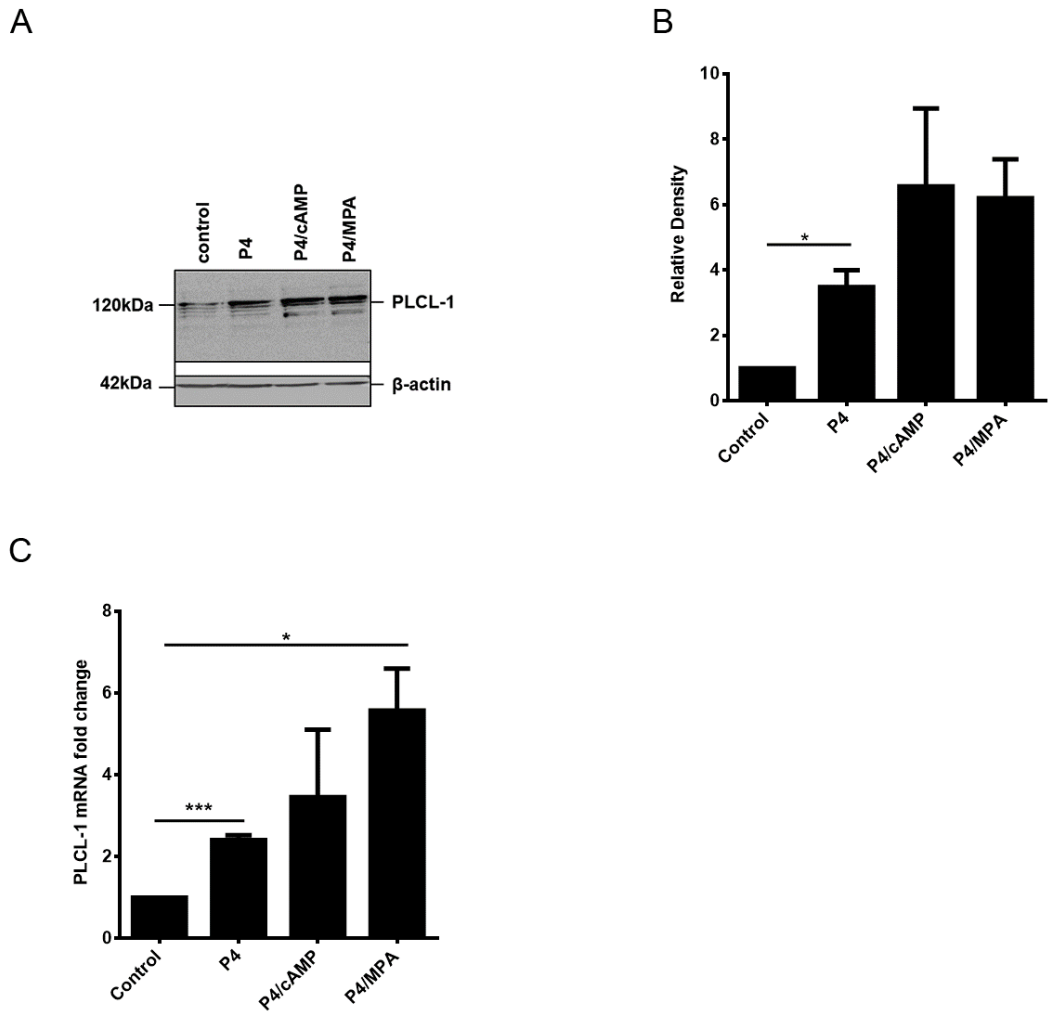

Figure 6.2.1b- ***Effect of P4 on PLCL-1 Expression*****.** Primary myometrium cells were serum starved for 24 hours and treated with either, P4, P4/cAMP or P4 and MPA together. A) Cells were harvested after 96 hours of treatment and cell lysates were analysed for PLCL-1 protein expression by Western blot. B) Band intensity from western blot was calculated and treated samples compared relative to control. C) mRNA fold change of PLCL-1 was analysed using RT-PCR. Data was analysed using one-way ANOVA with Dunnett's post hoc test. MPA and cAMP/MPA treated cells were compared to control, n=3, $P \leq 0.05$ (*).

6.2.2- Identifying what Region Within the Promoter of the PLCL-1 Locus Undergoes Increased Chromatin Accessibility upon Progesterone Treatment

Assay for Transposase Accessible Chromatin with high-throughput sequencing (ATAC-seq) is a technique used to outline chromatin accessibility. The ATAC-seq method utilises the actions of the transposase Tn5 enzyme. Tn5 is an enzyme that catalyses the movement of transposable elements (TE) from one part of the genome to another. In ATAC-seq Tn5 is used to create a double stranded break in areas of the genome that are highly exposed or have increased accessibility. Adapters are then added to be ligated into the cut DNA. The genome is then fragmented, and the adapter ligated fragments are isolated, and PCR amplified for sequencing. Areas in the genome that are more open and accessible will have more adapters ligated into them, these areas will have more reads upon sequencing, and hence ATAC-seq is used to map chromatin accessibility (Figure 6.2.2a)

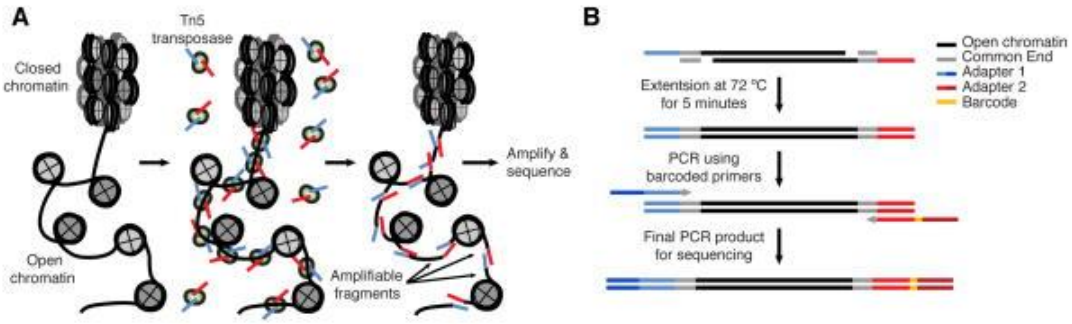

Figure 6.2.2a – *ATAC-sequencing Library* (A)Schematic representation of ATAC-seq library preparation method**.** DNA is simultaneously fragmented and tagged with sequencing adapters. (B) The DNA fragments are purified and preceding amplification, adapters are extended with a 72°C extension step. Additional sequence is incorporated into the adapters during the PCR, which comprise common sequencing ends and a sequencing barcode. Figure from Buenrostro et al, 2015.

Dr Raffaella Lucciola, PhD (University of Warwick) used ATAC-sequencing to map regions with increased chromatin accessibility when human endometrial stromal cells were decidualised with MPA and cAMP for 96 hours. The ATAC-seq data was utilised to identify regions near the PLCL-1 locus that increased chromatin accessibility upon treatment with MPA. Even though the cell type used for the ATAC-seq data was HESCs and not MSMCs, numerous peaks were identified around the PLCL-1 locus, which showed increased chromatin accessibility when endometrial stromal cells were treated with MPA and cAMP, as opposed to untreated cells. The ATAC-seq data was used to identify which target region to design the ChIP-qPCR primers for in myometrial cells.

Endometrial stromal cells were decidualised for 96 hours using MPA and cAMP. Decidualised endometrial stromal cells and undifferentiated stromal cells were prepared for ATAC-seq analysis. Illumina Hiseq-2500 was used to sequence the PCR amplified fragments giving a sequencing depth of 30 million paired end reads, with 100bp per fragment. The reads were mapped to the h19 genome using bowtie2-2.2.6 and samtools-1.2.0. Peak calling was performed using MACS-2.1.0; 202,169 peaks were identified and HTSeq-0.6.1 (Anders *et al*, 2015) was used to count the reads overlapping the peaks and DESeq2 was used to find differential peaks (Zhang *et al*, 2008) (Anders and Hubel, 2010).

Numerous peaks were identified around the PLCL-1 locus, which showed increased chromatin accessibility when endometrial stromal cells were treated with MPA and

cAMP, as opposed to untreated cells. A 261bp region (chr2:198,667,819-198,668,079) within the promoter of the PLCL-1 gene was found to have high chromatin accessibility when endometrial stromal cells were treated with MPA and cAMP. The 261bp region (chr2:198,667,819-198,668,079) was found to be significant using MACS 2 (P ≤0.05, N=3) when comparing MPA treated cells to control cells (Zhang *et al*, 2008). Thereafter, the q value is found from the p value using the Benjamini-Hochberg procedure. The q value is the FDR (false discovery rate) corrected p value, the empirical FDR is defined as number of control peaks / number of decidualised peak. The 261bp region (chr2:198,667,819-198,668,079) was found to be still significant after correction for the FDR using the Benjamini-Hochberg procedure ($q \leq 1 \times 10^{-4}$, n=3) (Figure 6.2.2b).

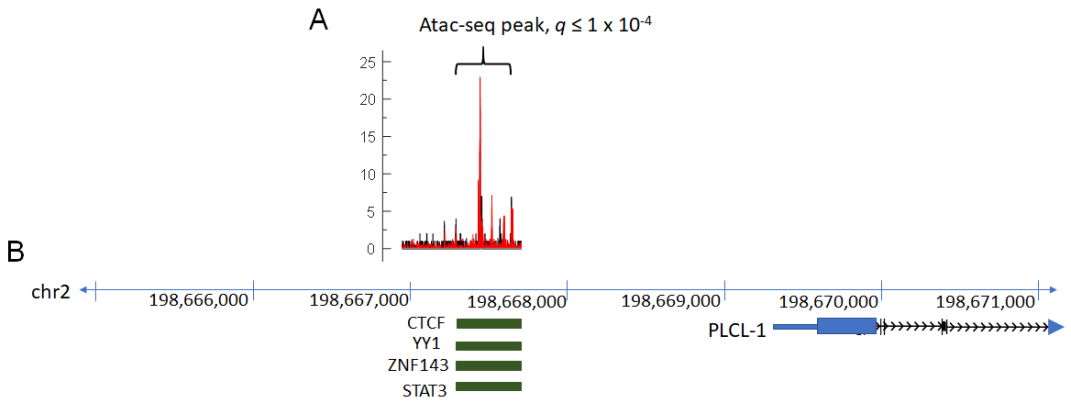

Figure 6.2.2b-*Assessing Chromatin Accessibility of PLCL-1 Locus upon Decidualisation*. Endometrial stromal cells were decidualised for 96 hours using MPA and cAMP, cells were then used for ATAC-seq analysis. A) Peaks represent level of chromatin accessibility, red peaks are from decidualised cells, black peaks represent undifferentiated stromal cells. The higher the peak the more accessible that DNA region is. The brackets identify the region of interest with its q value. B) Schematic representation of the PLCL-1 gene showing where it is located in chromosome 2, and the transcription factors that bind to the ATAC-seq identified region.

6.2.3- Using ChIP-qPCR in Primary MSMCs and Myla cells, to Study Differences in Transcription Factor Binding to the ATAC-seq Identified 261bp region, upon Treatment with cAMP/MPA

Chromatin immunoprecipitation is a technique that is used to study protein-DNA interactions. The DNA and protein are crosslinked using formaldehyde, the cells are lysed, and the DNA is sheared using a sonicator. Part of the sheared DNA is used as an input control; the rest is used for immunoprecipitation using specific antibodies. Non-specific binding is removed using washing, the cross-linking is then reversed, and the DNA is purified for qPCR.

Genome browser and ENCODE was used to find transcription factor binding motif within the 261bp region that was identified by Atac-seq. The region was found to be bound by 4 transcriptions factors: STAT3, CTCF,YY1 and ZNF143. ChIP-antibodies specific to the 4 transcription factors were used for ChIP-qPCR. Myla and primary myometrial cells were serum starved and treated with either cAMP, MPA or cAMP/MPA for 96 before harvesting for ChIP-qPCR. Forward and reverse primers (Table 2.3) spanning the 261bp were designed to study differences in transcription factor binding to this region after cAMP/MPA treatment. ChIP-qPCR data was normalised using the Percentage Input method; input represents the total amount of chromatin that is used before immunoprecipitation. The CT values from the CHIP are calculated as a percentage of the CT values from the input.

In Myla cells, using a CTCF specific antibody, there was an increase in mean percentage input in cells treated with cAMP, MPA and cAMP/MPA (2.91 and 3.63, and 3.40, respectively) compared to control (1.56). Using a YY1 and ZNF143 specific antibodies a similar trend in mean percentage input as seen in CTCF samples (Figure 6.2.3a). The STAT3 antibody did not give an adequate DNA yield for qPCR.

ChIP-qPCR experiments were repeated in primary myometrial cells using the antibodies CTCF, YY1 and ZNF143. Using a CTCF specific antibody, the percentage input increased in cells treated with MPA compared to control. MPA treated cells had a percentage input of 2.23 compared to 1.2 in control samples. ChIP-qPCR with ZNF143 and YY1 antibody in primary cells showed an increased percentage input when cells were treated with cAMP, MPA and cAMP/MPA compared to control, with cAMP/MPA exhibiting the largest increase (Figure 6.2.3b).

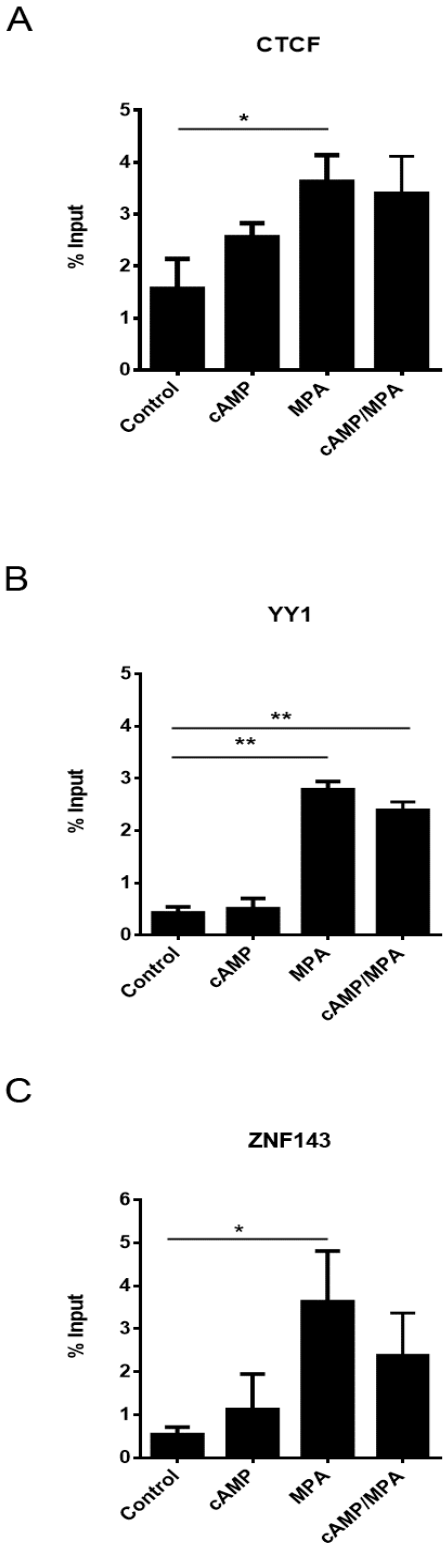

Figure 6.2.3a- ***ChIP-qPCR of Myla Cells Using CTCF (A),YY1(B) and ZNF143 (C) Antibodies*****.** Myla cells were serum starved for 24 hours before treatment with cAMP, MPA or cAMP/MPA for 96 hours. Cells were then prepped for ChIP-qPCR; data was analysed using Percentage Input Method. Data was analysed using one-way ANOVA with Dunnett's post-hoc test, *P* ≤0.05 (*), n=3, error bars represent standard deviation.

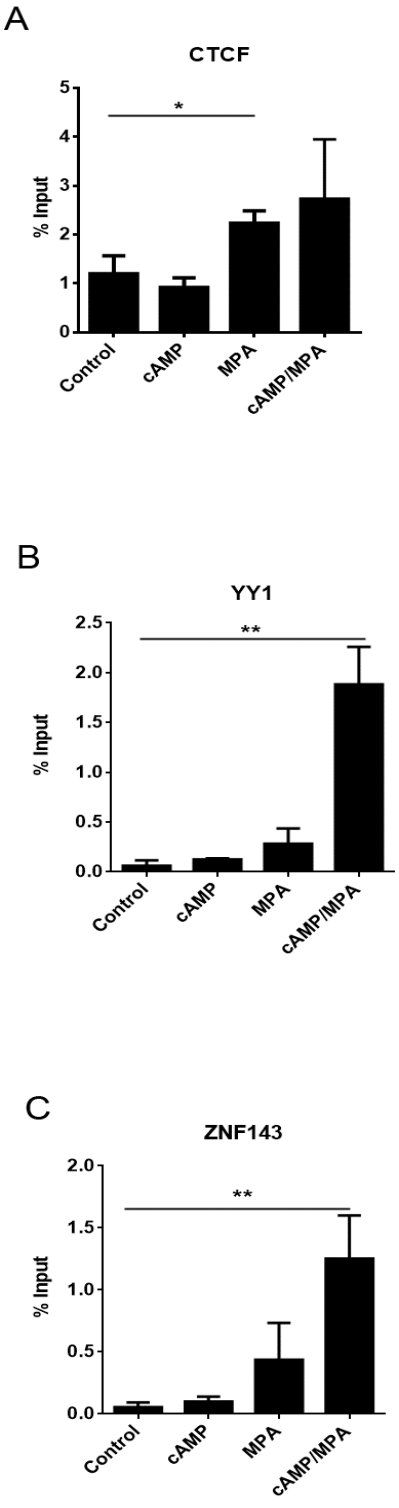

Figure 6.2.3b -*ChIP-qPCR of Primary Myometrial Cells Using CTCF (A) ,YY1(B) and ZNF143 (C) Antibodies.* Primary myometrial cells were serum starved for 24 hours before treatment with cAMP, MPA or cAMP/MPA for 96 hours. Cells were then prepped for ChIP-qPCR; data was analysed using Percentage Input Method. Data was analysed using one-way ANOVA with Dunnett's post-hoc test, p ≤0.05p (*), n=3, error bars represent standard deviation

6.2.4- Is PLCL-1 mRNA Detectable in Whole Blood from Mid-gestation to Late Gestation and Active Labour?

Whole blood samples were collected from pregnant women to investigate if PLCL-1 expression is measurable from blood. Samples were collected from women in their second trimester (mid-gestation) and women who were in active spontaneous labour. The women in their second trimester were pregnant from 16-22 weeks. RNA was extracted from the whole blood, this was then converted to cDNA for quantitative PCR. The CT values of PLCL-1 and the housekeeping gene L19 were analysed. Cycle threshold (CT) is characterised as the number of cycles needed for the fluorescent signal to exceed the background fluorescent signal. CT values are inversely proportional to the quantity of target mRNA in the sample. The PLCL-1 CT values were normalised against the housekeeping gene (Figure 6.2.4). Whole blood samples from women in their second trimester had a mean normalised CT value of 7.25 (± SD 1.01), which decreased slightly to 7.02 (± SD 0.42) for IL samples. This decrease was not found to be significant when using Student's t test. Cell free RNA samples from women in their second trimester had a mean normalised CT value of 10.19 (± SD 0.70), which decreases to 8.30 (± SD 0.62) for IL samples. This decrease was not found to be significant when using Student's t test.

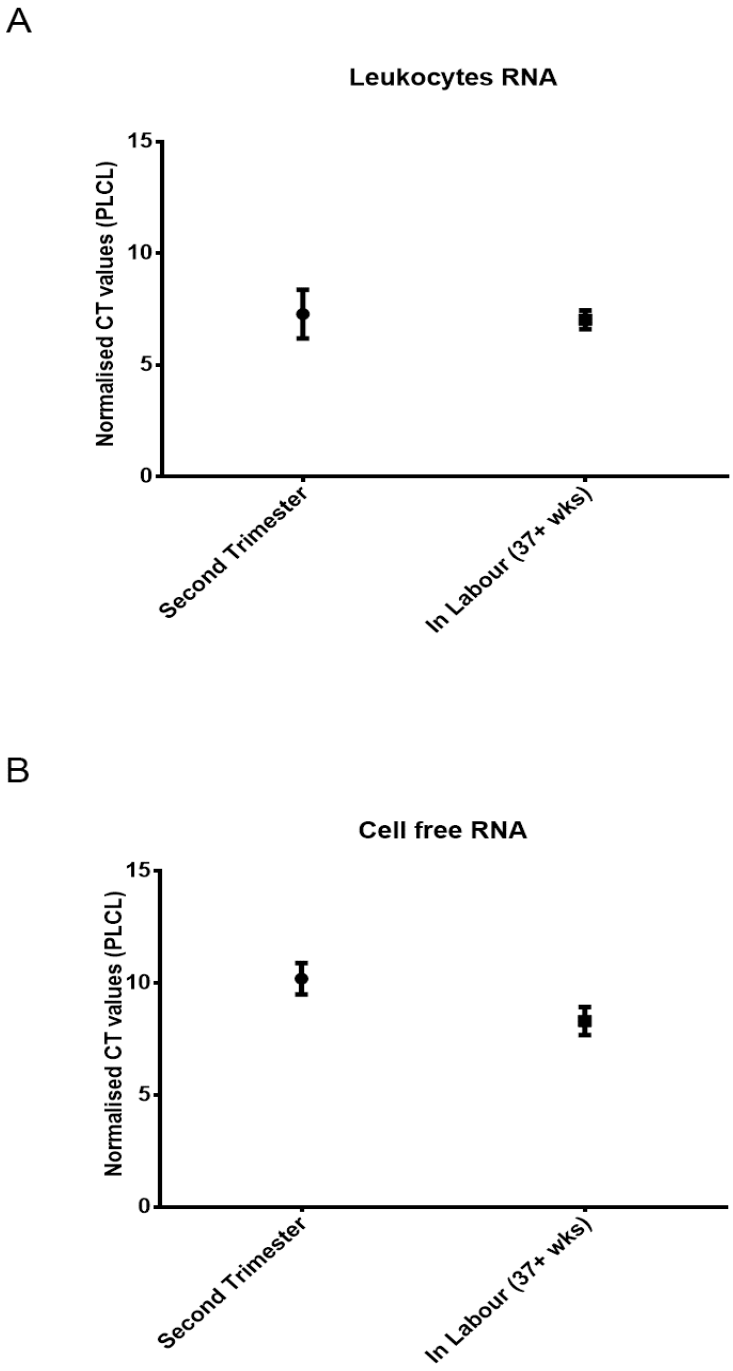

Figure 6.2.4-Expression of PLCL-1 in the Blood . Whole blood (A) or plasma (B) was collected from women who were in their second trimester and women in active spontaneous term labour (+37 weeks). RNA was extracted from for RT-PCR. PLCL-1, L19 mRNA levels were detected in RT-PCR. PLCL-1 CT was normalised against housekeeping gene (L19). Data was analysed using Student's t-test, $P \leq 0.05$ (*). , n=3, error bars represent standard deviation.

6.3 Discussion

MPA and P4 both Increase Expression of PLCL-1

Medroxyprogesterone acetate (MPA), is a synthetic progestin. MPA binds to and activates PRs, MPA has been shown to have a stronger binding affinity for PRs compared to P4 (Killinger *et al*, 1985) (Schindler *et al*, 2003) (Kuhl, 2005). MPA significantly increased expression of PLCL-1, suggesting that PR activation causes a rise in PLCL-1 transcription and translation. However, MPA has a weak affinity for androgenic receptor (AR), which P4 does not (Bentel *et al*, 1999) (Phillips *et al*, 1987) (Kuhl, 2005) . Additionally, MPA has a stronger affinity for glucocorticoid receptors (GR) and mineralocorticoid receptor (MR) than P4 (Bamberger *et al*, 1999) (Kontula *et al*, 1983) (Kuhl,2005). Thus, it may be that effects of MPA on PLCL-1 expression possibly be through its agonistic actions on AR, GR and MR and not through its actions on PRs. Therefore, MSMCs were treated with P4, which also showed an increase in PLCL-1 expression, this shows that the increase in PLCL-1 expression by MPA and P4 is likely to be due to the actions of progesterone receptor.

Co-treatment of cAMP and P4/MPA Increases Expression of PLCL-1

Cyclic AMP increased expression of PLCL-1, although not as much as P4 or MPA. Cyclic AMP has been shown to regulate transcription factors through its activation of PKA (Table 6.3.1). Activated PKA translocates to the nucleus, whereby it phosphorylates transcription factors, which bind to cAMP response elements (CRE) within the promoter of target genes. Many genes that modulate myometrial contractility have

been identified that respond to cAMP (Table 6.3.1). The increase in PLCL-1 expression by cAMP, suggest that PLCL-1 transcription is regulated through the cAMP/PKA pathway. However, the augmented effects of cAMP/P4 or cAMP/MPA on PLCL-1 expression, suggest that it's through the combined effects of cAMP and progesterone that PLCL-1 expression is regulated, rather than cAMP alone.

Table 6.3.1- ***cAMP Facilitates Muscle Relaxation by Changing Gene Expression profile.***

Signalling Pathway	*Cell type*	*Results*	*References*
cAMP/PKA/CREB	Human myometrial cells	- Forskolin increased cAMP, PKA and phospho-CREB levels. - Forskolin reduced OXTR mRNA. - Knockdown of PKA, reduced forskolin induced reduction of OTR.	(Yulia *et al*, 2016)
cAMP/PKA P4	Human myometrial cells	- Forskolin reduced expression of OXTR, Cx43 and *PTGFR*. - P4 amplified the forskolin induced reduction of - cAMP/PKA signalling pathway work synergistically with P4 to reduce expression of CAPs.	(Amini *et al*, 2019)
cAMP/PKA P4	Human myometrial cells	- cAMP reduced NF-κB DNA binding. - cAMP amplified effects of P4 on gene expression. - Increases expression .	(Chen *et al*, 2014)
cAMP/PKA	Airway smooth muscle cells	- cAMP reduced NF-κb DNA binding. - cAMP reduced expression of IL-8.	(Oldenburger *et al*, 2012)

There are many examples of cAMP and PR working synergistically and there is emerging evidence suggesting that the cAMP pathway integrates with the P4 pathway and gives cellular specificity to progesterone signalling, mainly by regulating transcription factors that modulate PR function (Amini *et al*, 2019) (Chen *et al*, 2014). The cross talk between the cAMP pathway and the PR pathway is most evident in the decidualisation of the endometrium (Brar *et al*, 1997) (Gellersen and Brosens, 2005). Decidualisation is the differentiation of endometrial stromal cells (ESCs), whereby they take on a secretory phenotype and undergo morphological changes (Gellersen and Brosens, 2005). Progesterone acting on ESCs is vital for ECSs to undergo decidualisation, and one of the peptides secreted by differentiated ESCs is prolactin (PRL), which is used a marker for decidualisation (Brosens *et al*,1999) (Tellgman and Gellersen, 1998). A combined treatment of HESCs with MPA and cAMP, increases the expression of PRL (Brosens *et al*, 1999). Pre-treatment of HESCs with cAMP before the addition of MPA, accelerated the transcription of PRL, suggesting the cAMP/PKA pathway sensitised cells for progestin actions, indicating a synergism between cAMP/PKA and P4/PR (Brosens *et al*, 1999).

Activated PKA translocates to the nucleus and phosphorylates transcriptional regulators CREB and CREM (Gonzalez *et al*, 1989) (Hagiwara *et al*, 1993). Phosphorylated CREB/CREM recruit CREB binding protein (CBP), which has intrinsic histone acetyltransferase activity causing it to remodel chromatin structure for increased DNA accessibility, leading to increased gene expression (Kwok *et al*, 1994) (Parker *et al*, 1996). CBP also recruits other transcription factors such as the

CCAAT/enhancer-binding protein (C/EBP)(Mink *et al*, 1997). The PRL promoter has both C/EBP binding sites and PR half sites, and PR can bind to C/EBP (Pohnke *et al*, 1999). It may be that C/EBP acts as a tethering protein for PR to bind to the promoter of the PRL gene; illustrating the mechanism by which cAMP and P4 work synergistically to upregulate expression of PRL. It could also be the same mechanism that cAMP/P4 employ to increase chromatin accessibility near the PLCL-1 locus, resulting in increases expression of PLCL-1. In addition to C/EBP; STAT5 and FOXO1a are transcription factors induced by cAMP and are capable of binding to PR (Gellersen and Brosens, 2005). Additionally, CBP is recruited to the pre-initiation complex by the PR co-activator -1. The interaction of the cAMP inducible transcription factors with PR, creates a model for the cross talk between cAMP and PR, which regulates gene expression and may be the model that causes an increase in PLCL-1 expression in MSMCs upon treatment with cAMP/MPA/P4 (Figure 6.3.2).

In addition to the evidence in HESCs showing the cross talk between cAMP and progesterone, there is also evidence that this cross talk exists in the myometrium and it is important for myometrial quiescence. In human MSMCs, cAMP reduces expression of the CAP genes: OXTR, Cx43 and PTGFR. Cotreatment of cAMP and P4 led a further reduction in expression of OXTR, Cx43 and PTGFR (Amini *et al*, 2019). Chen et al, showed that cAMP increased PRB binding to PRE in DNA and augmented progesterone's suppression of COX-2 expression and progesterone's induced expression of FKBP5. (Chen *et al*, 2014).

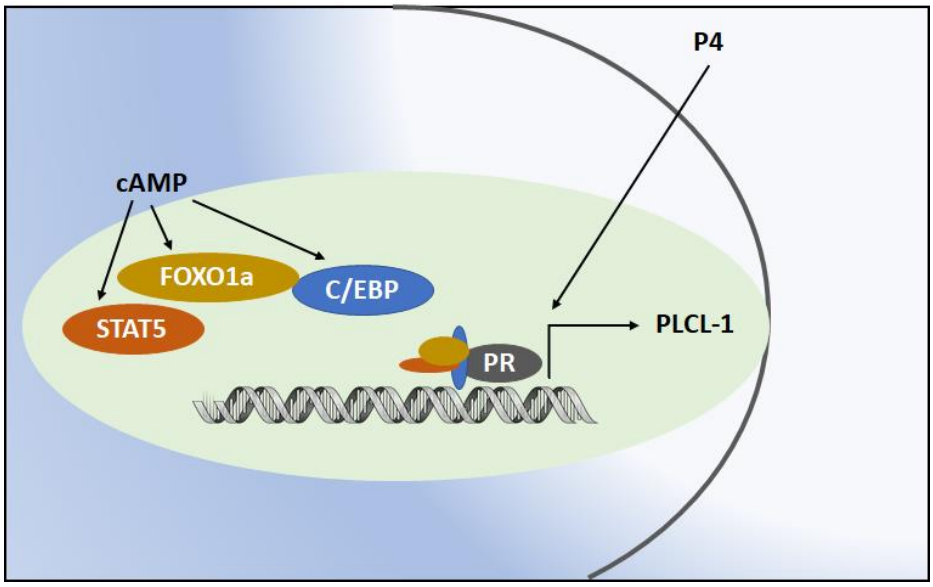

Figure 6.3.2- ***Proposed Model of cAMP/P4 Synergism to Increase Expression of PLCL-1.*** Elevated levels of camp leads to increased activation of camp responsive transcription factors (C/EBP, STAT5 and F0XO1a) which interact with PR to increase transcription of PLCL-1.

cAMP/MPA Treatment of HESCs Increases DNA Accessibility of PLCL-1 Promoter and Transcription Factor Binding in MSMCs

Treatment of HESCs with cAMP/MPA led to increased chromatin accessibility of a 261bp region (chr2:198,667,819-198,668,079) near the PLCL-1 locus. The increased chromatin accessibility of this region shows that upon cAMP/MPA treatment the PLCL-1 promoter becomes accessible for protein binding and transcriptional activation.

ChIP-qPCR showed a significant increase in CTCF binding to the 261bp region upon treatment with cAMP/MPA in both primary MSMCs and Myla cells. CTCF is an 82 kDa protein that functions as a ubiquitously expressed transcriptional regulator (Filippova *et al*, 1996). It recognises and binds to the DNA sequence CCCTC, and there are around 55,000 CTCF binding regions in the genome; 50% of the sites are in non-coding regions, 35% are in within gene sequences and 15% are proximal to promoter sites (Chen *et al,* 2008) (Chen *et al*, 2012). CTCF has a conserved structure, that is separated into 3 different domains; N-terminal region, C-terminal region and central domain (Vostrov *et al*, 2002). The central domain has 11 zinc fingers, which CTCF uses to bind to DNA. CTCF is a multi-functional protein that recruits different binding proteins that can regulate the function of CTCF.

Studies show that CTFC functions as either a transcriptional repressor or activator or an insulator protein (Filippova *et al*, 1996)(Gaszner *et al*, 2006) (Klenova *et al*, 1993) . Insulator sequences are short sequences found between enhancer sequence and gene promoters. CTCF can bind insulators sequences to impede any interaction between the enhancers and promoters, leading to gene repression. In the chicken β-globin locus; CTCF was found to bind to insulator sequences, causing the formation of a chromatin loop that blocked any access to enhancer sequences, causing a repression of β-globin expression (Splinter *et al*, 2006). However, CTCF can also form a chromatin loop, so that the enhancer and the promoter can interact; there is transcriptional activation (Kim *et al*, 2015).

CTCF co-localises with cohesin in the genome. Cohesin is a ring-shaped protein, made up of three subunits: SMC1, SMC3 and SCC3. Cohesin forms a ring around chromatin loops, to stabilize it and increase interactions between enhancers and promoters, allowing transcription factors to bind to enhancers and be near promoters for transcriptional activation (Kagey *et al*, 2010) (Parelho *et al*, 2008) (Wendt *et al*, 2008) (Figure 6.3.3). Cohesin does not directly bind to DNA, it functions through its association with CTCF. ChIP-qPCR studies showed that alongside CTCF, there was binding of SMC3 and RAD21 (component of the cohesin complex) on the 261bp region near the PLCL-1 locus.

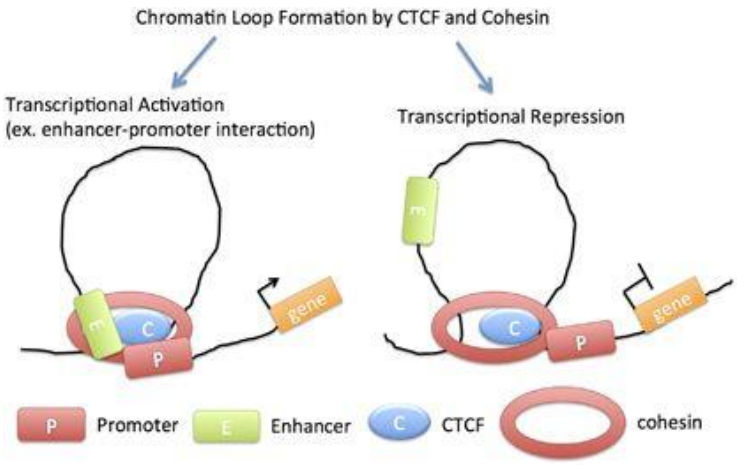

Figure 6.3.3- ***Interaction Between CTCF and Cohesin***. CTCF and cohesin complex cause either activate or inhibit transcription. Cohesin and CTCF can create a chromatin loop that contains enhancers and promoters, leading to transcriptional activation. A chromatin loop that foils the enhancer from interacting with the promoter leads to inhibition of transcription. Figure from Kim *et al*, 2015

CTCF is regulated by post-translational modifications and by its binding partners (Weth *et al*, 2011). One binding partner of CTCF is Yin and yang 1 (YY1), which also had a significant increase in binding to the 261bp region near the PLCL-1 locus, upon

treatment with cAMP/MPA in both primary MSMCs and Myla cells (Donohoe *et al*, 2007). YY1 is a transcription factor that acts as both an activator and repressor (Gordon *et al*, 2006). Co-immunoprecipitation studies show that YY1 binds to the N-terminus domain of CTCF. The interaction between YY1 and CTCF is particularly important for X-inactivation(Donohoe *et al*, 2007). YY1 and CTCF associate together to amplify transcription of Xist; a non-coding antisense RNA that is involved in mammalian X-inactivation (Donohoe *et al*, 2007). As YY1 and CTCF binding can lead to enhanced transcriptional activation, it may be that treatment of MSMCs with P4, leads to increased recruitment of YY1/CTCF to the PLCL-1 promoters leading to increased transcription of PLCL-1.

Zinc finger protein 143 (ZNF143) is a transcriptional activator, that was first discovered activating transcription of the tRNA gene, through binding its promoter and recruiting RNA polymerase III (Schaub *et al*,1999) (Hernandez-Negrete *et al*, 2011). Treatment of MSMCs with cAMP/MPA increased ZNF143 binding near the PLCL-1 locus. Apart from its role as a transcriptional activator, ZNF143 has also been implicated in formation of chromatin loops; to provide proximity between distal enhancer sequences to promoter regions, leading to increased gene expression. Bailey *et al* proposed a model, whereby through the interactions between ZNF143, CTCF, RNA pol 2 and cohesin, there is interaction between distal promoters and enhancer sequence (Bailer *et al*, 2015) (Figure 6.3.4).

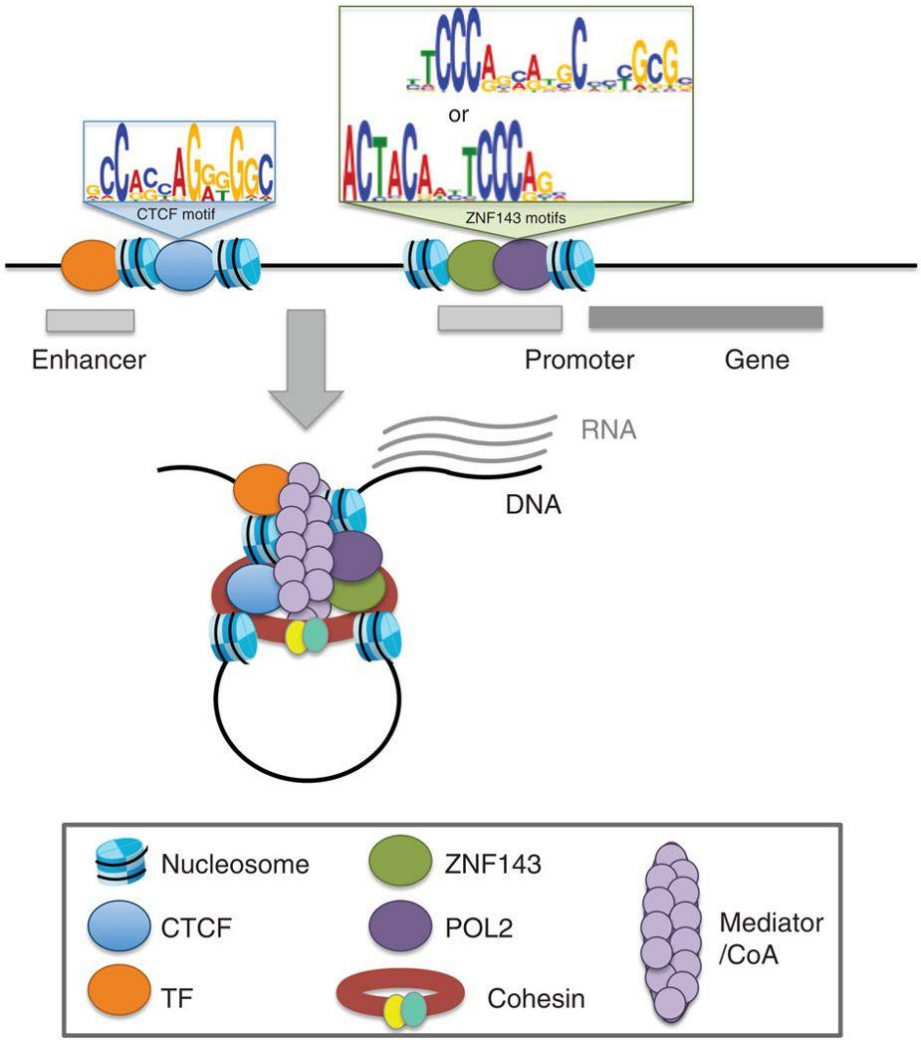

Figure 6.3.4- ***Proposed Model for Interactions Between ZNF143 and CTFC, and Other Binding Proteins.*** ZNF143 binds directly to promoters, whilst CTCF binds to insulator sequences near enhancers. The interaction between ZNF143 and CTCF, bring enhancer sequences and promoter sequence near to each other. Allowing transcription factors (TF) that are bound to enhancers to activate transcription of genes by recruiting RNA polymerases (POL2) to the promoters. Figure from Bailey *et al*, 2015

Chapter 7

General Discussion

7.1- The Challenges of Researching and Preventing Preterm Labour

The complexity and uniqueness of human parturition in comparison to the available animal models renders it very challenging to study the mechanism of labour and preterm labour. There are still many questions and gaps in the knowledge of the different phases of pregnancy and parturition; starting from implantation through to involution, and the differences between term and preterm labour. Perhaps preterm labour is just an accelerated form of term labour and the mechanism that lead to both is the same, but an environmental or genetic factor triggers an acceleration of the biochemical pathways to parturition; causing premature labour. Another explanation is that there is a clear difference in the molecular pathways between term and preterm labour, and it is the activation of a specific pathway that causes premature labour.

A tangible explanation to the mechanism of term and preterm labour in humans will require a suitable model, in which the findings can then be confidently translatable to humans. Currently the different animal models for studying parturition are limiting due to the uniqueness of human parturition. In sheep, parturition is initiated by foetal signals that derive from the HPA axis (Liggins *et al*, 1967) (Liggins *et al*, 1968). In goats, parturition follows the dissolution of the corpus luteum (Cooke *et al*, 1980). Comparative genomic studies between humans and chimpanzees, show a genome that is 95% similar; the greatest difference being in genes that govern reproduction (Chen *et al*, 2005) (Chimpanzee Sequencing and Analysis Consortium,2005). Additionally, the changes in the female pelvis, due to the evolving of humans into an

upright position and the relative large human cranium have had consequences for human parturition. It could be, that the large differences in human parturition in comparison to other mammals is due the distinctiveness of the human pelvis.

Undoubtedly the main obstacle in using animal models to study human parturition is that most of the animal models, undergo a withdrawal of progesterone in the maternal systemic circulation. In humans there is no such withdrawal in circulatory progesterone, but the hypothesis of a functional progesterone withdrawal, which has different proposed mechanisms. Functional progesterone withdrawal rather than circulatory progesterone withdrawal may explain why progesterone is a weak tocolytic in humans (O'Brien *et al*, 2007) (Rouse *et al*, 2007) (Kuon *et al*, 2010). The continuous rise in preterm labour globally highlights the urgent need for better diagnostic tools and more effective tocolytics; this will only be achieved by using animal models that have a parturition process that is more similar to humans and a better understanding of the mechanism of progesterone and its role in human pregnancy.

Any potential new tocolytic will have to consider the issue of gene redundancy in parturition for it to be effective at improving neonatal outcomes. Gene redundancy is when there are several different genes that perform the same biological function, thus a loss in the expression of one gene will have no consequence on the phenotype (Nowak *et al*, 1997). A series of experiments on transgenic mice demonstrate the genetic redundancy in biological pathways that mediate parturition. In mice, a reduction in progesterone due to luteolysis initiates labour, and luteolysis is instigated by a rise in pre-partum $PGF_{2\alpha}$ (Uozumi *et al*,1997) (Sugimoto *et al*, 1997)

(Bonventre *et al*, 1997). In the myometrium, $PGF_{2\alpha}$ and OT both bind to $G_{\alpha q}$ coupled GPCRs; activating the same biological cascade, which leads to contraction (Blanks *et al*, 2007) (Lopez-Bernal,2003) (Sanborn *et al*, 2005). OT null mice undergo normal delivery of offspring, whilst COX-1 null mice fail to deliver until surgical or pharmacological intervention produces luteolysis and subsequent progesterone withdrawal, re-establishing normal delivery (Nishimori *et al*, 1996) (Young *et al*, 1996). Therefore, the transgenic mice show that a loss in either OT or prostaglandins (PG) does not stop contraction after progesterone withdrawal has begun. In double knockout mice (OT/ COX-1 null) luteolysis does occur and labour is initiated but the mice experience prolonged delivery. As labour is not triggered in COX-1 null mice but does in double knock out mice, it shows that there is compensation when OT is knocked out and that the OT pathway has luteotrophic functions that is only unveiled in the double knockout mice (Gross *et al*, 1998). Treatment of OT null mice with OT, show that the OT null mice are more sensitive to OT compared the wild type (WT) mice, and at high OT dosage both OT null and WT mice undergo premature labour without luteolysis and progesterone withdrawal (Imamura *et al*, 2000). The increased sensitivity of OT null mice to OT, suggest an upregulation of OTR receptors in OT null mice compared to WT. The insertion of a neomycin cassette in COX-1 gene created a COX-1 knockdown mice, resulting in a reduction in production of PG but normal parturition, even though the levels of PG were similar to COX-1 null mice, which could not initiate labour. OTR was upregulated in the COX-1 knockdown mice, this shows that there is compensation between the OT pathway and the PG pathway, which suggest that there is gene redundancy in these two pathways (Yu *et al*, 2005).

Therefore, any novel tocolytic might have to have to target multiple proteins to bypass the genetic redundancy of parturition and prevent premature labour.

This thesis showed the role of PLCL-1 in uterine quiescence, an investigation prompted due to its reduced expression in labouring myometrium and is ability to inhibit release of $[Ca^{2+}]_i$ in neuronal cells.

To summarise, I provide data demonstrating that:

i. PLCL-1 regulates intracellular calcium concentrations in primary myometrial smooth muscle cells. The oxytocin induced rise in $[Ca^{2+}]_i$ is amplified with the loss of PLCL-1 expression or subdued with the overexpression of PLCL-1.
ii. PLCL-1 mediates changes in the transcriptome of primary MSMCS; directly affecting expression of CAPs or quiescent genes.
iii. Through the inhibition of phosphodiesterases, PLCL-1 can maintain basal cAMP levels.
iv. Progesterone, MPA and cAMP all increase expression of PLCL-1. MPA also changes the chromatin landscape of the PLCL-1 gene locus making it more accessible for induction of gene expression.
v. Progesterone recruits co-factors to PLCL-1 locus; illustrating a mechanism whereby progesterone is able induce expression of PLCL-1.

7.2-PLCL-1 Decreases Myometrial Contractility

PLCL-1 decreases myometrial contractility by regulating the contractile apparatus of the MSMC; PLCL-1 does this both directly and indirectly. Calcium is the central molecule for contraction of the smooth muscle cells, the rise $[Ca^{2+}]_i$ leads to a cascade of substrate activation, eventually resulting in cross-bridge cycling (contraction-relaxation). PLCL-1 directly inhibits cross-bridge cycling by inhibiting the rise $[Ca^{2+}]_i$, hence directly preventing contraction in MSMCs. PLCL-1 indirectly inhibits myometrial contractions by maintaining cAMP concentration, through its inhibition of PDE7B. Cyclic AMP activates PKA, which then inhibits MLCK. PLCL-1 indirectly promotes MSMCs quiescence by regulating the cAMP/PKA/MLCK pathway.

Another explanation for the PLCL-1 mediated reduction in $[Ca^{2+}]_i$ could be through the binding of PLCL-1 to phosphatases PP1c and PP2A. PP1c is involved in the regulation of calcium release from ER. PPD2 is part of peptide group that selectively activate different phosphatases, by disruption the binding of phosphates to proteins that inhibit their activity (Chatterjee *et al*, 2012). Treating Hela cells with PPD2 causes a spike in calcium oscillations. The increase in $[Ca^{2+}]_i$ in Hela by PPD2 was due to release from intracellular calcium stores, as the experiments were conducted without the presence of external calcium. Furthermore, the increase in $[Ca^{2+}]_i$ by PPD2 was mediated by its actions on IP_3 receptors on the endoplasmic reticulum (ER). Using IP_3 receptor selective inhibitor Xestospongin C, PPD2 mediated increase in $[Ca^{2+}]_i$ was lost (Reither *et al*, 2013).

Phosphatases also control entry of external calcium into smooth muscle cells, by regulating L-type VOCC. Tautomycin: a potent and selective PP1/PP2a inhibitor (Cheng *et al*, 1987) (MacKintosh and Klumpp, 1990), was shown to inhibit the opening of L-VOCC, this signifies that dephosphorylation of L - VOCC by PP1/PP2a increases its activity (Groschner *et al*, 1995) (Figure 7.2.1) However this study was opposed when the purified catalytic subunit of PP2A (PP2ac), inhibited the opening of L-VOCC, whilst the catalytic subunit of PP1 (PP1c) had no effect the opening of L-VOCC (Groschner *et al*, 1996). Both studies were conducted in primary vascular smooth muscle cells from human umbilical veins (Groschner *et al*, 1995, 1996).

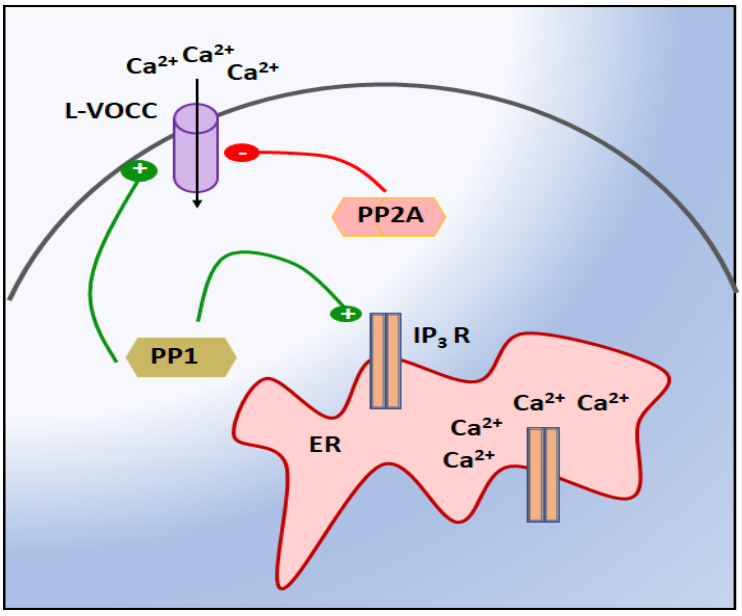

Figure 7.2.1-*Modulation of L-VOCC and IP3R by Phosphatases.* Protein phosphatase 1 (PP1) dephosphorylates L-type voltage operated calcium channels (L-VOCC) and this may increase the open state duration of L-VOCC. Paradoxically dephosphorylation of L-VOCC by protein phosphatase 2a (PP2a) has an opposite effect on L-VOCC compared to PP1. Dephosphorylation of IP3 receptors (IP3R) on the endoplasmic reticulum by PP1, modulates the opening of IP3R .

PLCL-1 binds to the catalytic subunits of the phosphatases: PP1 and PP2a (Figure 7.2.2). (Yoshimura *et al*, 2001) (Kanematsu *et al*, 2006). The binding sites on PLCL-1 for PP1 and PP2a are close, however binding of the two phosphatases is mutually exclusive, as PP2a binding to PLCL-1 results in PP1 to dissociate from PLCL-1. Similarly, PP1 causes PP2a to dissociate from PLCL-1 (Sugiyama *et al*, 2012). PLCL-1 affects the dephosphorylation activity of PP1 but not PP2A. PLCL-1 inhibited the ability of PP1 to dephosphorylate myosin light chain in a dose dependent manner, but no such effect was observed on the catalytic activity of PP2a (Yoshimura *et al*, 2001) (Sugiyama *et al*, 2012) (Terunuma *et al*, 2004).

PLCL-1 is a target protein for PKA phosphorylation. PKA phosphorylation of PLCL-1 resulted in the dissociation of PP1 from PLCL-1, however no similar effect was seen on PP2a (Terunuma *et al*, 2004) (Sugiyama *et al*, 2012). The dissociation of PP1 from PLCL-1 upon PKA phosphorylation of PLCL-1, increased the binding affinity of PP2a to PLCL-1 (Sugiyama *et al*, 2012). PKA phosphorylation of PLCL-1 increases activity of PP1; overexpressing PLCL-1 in PC12 cells accelerated SNAP-25 dephosphorylation rate by PP1 (Gao *et al*, 2012).

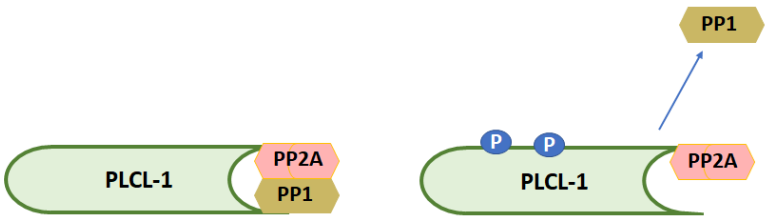

Figure 7.2.2-*PLCL-1 as a Scaffold Protein for Phospho-regulation*. PLCL-1 binds to both phosphatases PP2a and PP1. Phosphorylation of PLCL-1 causes the release of PP1, but increases PLCL-1 binding strength to PP2A.

The binding of both PP1 and PP2a to PLCL-1 and PLCL-1 being a target for PKA phosphorylation; suggest that PLCL-1 acts a scaffold protein that regulates phosphorylation states of other proteins (Sugiyama *et al*, 2013). The phosphorylation states of IP_3 receptor or L-VOCC can regulate their activity. As PLCL-1 may be a phospho-regulatory scaffold protein; PLCL-1 may indirectly regulate the phosphorylation states of IP_3 receptor and L-VOCC, and through this regulation it may explain the effects of PLCL-1 on $[Ca^{2+}]_i$ (Figure 7.2.3).

Activation of PP1 causes an increase in release of $[Ca^{2+}]_i$ from intracellular stores (Reither *et al*, 2013). PP1 also may increase the activity of L-VOCC. Overexpression of PLCL-1 decreases the activity of PP1, however phosphorylation of PLCL-1 causes PP1 to be released and this allows PP1 to dephosphorylate its target protein. It may be that PLCL-1 sequesters PP1 and inhibits PP1, and this may stop PP1 from increasing intracellular $[Ca^{2+}]_i$ via its actions on IP_3 receptors and L-VOCC.

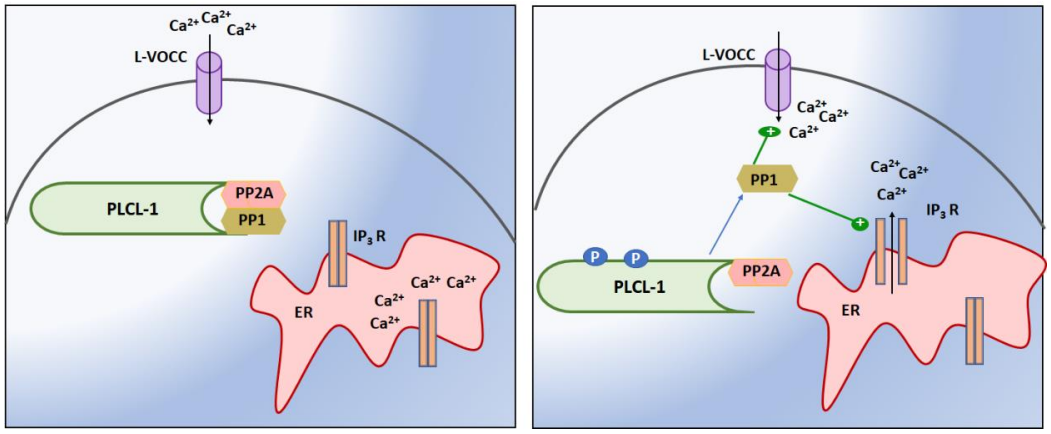

Figure 7.2.3 ***The Effect of PLCL-1's Role as Phospho-regulatory Protein on [Ca²⁺]i***. An unphosphorylated PLCL-1 sequesters both PP1 and PP2a. Phosphorylation of PLCL-1, allows the release of PP1, whose actions may lead to increased $[Ca^{2+}]$i.

7.3- Proposed Mechanisms for PLCL-1 Reduction in the Labouring Myometrium

PLCL-1 expression is modulated by progesterone (Chapter 6), The reduction of PLCL-1 in the labouring myometrium could be a consequence of the loss in progesterone signalling. One of the proposed models of `functional' progesterone withdrawal is that progesterone is synthesised and metabolised in a paracrine system. During the quiescence phase of pregnancy, progesterone is synthesised by the amnion, chorion and decidua where it then acts upon on the myometrium in a paracrine manner (Gib *et al*, 1980) (Mitchell *et al*, 1987). Before the onset of labour progesterone is then metabolised in the amnion, chorion into progesterone metabolites, keeping the circulatory maternal progesterone high, but low in the adjacent myometrium (Mahendroo *et al,* 1996) (Mitchell and Wong, 1993). PLCL-1 expression is increased by progesterone, however during labour there is a decrease in PLCL-1 expression in the myometrium. The PLCL-1 reduction in labouring myometrium, may be explained by local metabolism of progesterone in the foetal membrane and maternal decidua, (Figure 7.3). Another explanation for the localised myometrial reduction in PLCL-1, is the modulatory change in PR co-regulators. During the stimulation phase of pregnancy there is a inhibition of P4 co-activators in the myometrium, this leads to a loss in PR signalling, which may lead to a loss in PLCL-1 expression.

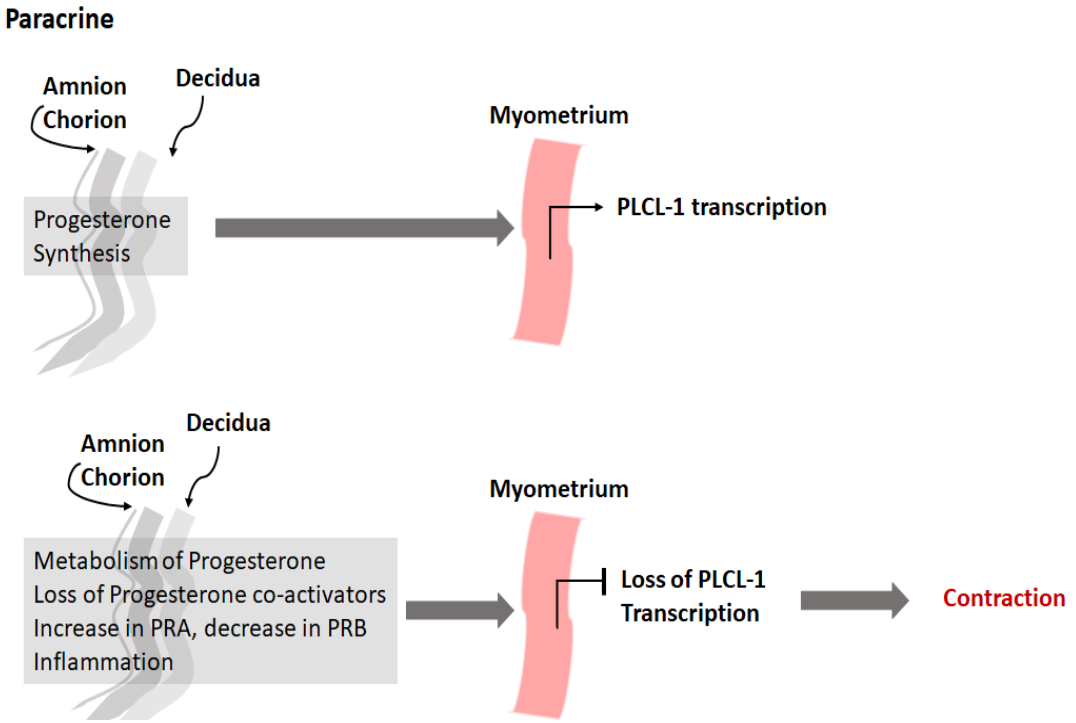

Figure 7.3- ***Proposed Model of Local Progesterone Functional Withdrawal Reducing PLCL-1 Expression*****.** Progesterone is synthesised and metabolised in a paracrine method, by the foetal membranes and the decidua. Progesterone or progesterone metabolites act on the neighbouring myometrium, leading to either transcription of PLCL-1 or no transcription of PLCL-1.

7.4- PLCL-1 Is an Effector Protein of Progesterone

ATAC-seq data, showed an increase in chromatin accessibility, proximate to the PLCL-1 locus upon decidualisation of HESCs. ATAC-seq data demonstrated that cAMP/MPA recruits histone acetyltransferases to the PLCL-1 promoter; permitting transcription factor binding to induce expression of PLCL-1. There are more than 300 co-regulators of PR. Co-regulators are sub-characterised as co-repressor or co-activators depending upon whether they inhibit or activate transcription, respectively.

Chromatin modifiers either increase chromatin accessibility or decrease it, this usually involves histone acetyltransferase (HAT) or histone deacetylases (HDAC), which modify chromatin conformation to either an open or closed state, respectively (Grunstein, 1997) (Strahl and Allis, 2000) (Brosens *et al*, 2004). An example of a co-regulator of PR is CREB binding protein (CBP) and steroid receptor co-activator 1 (SRC-1), which work synergistically to co-activate PR mediated transcription (Smith *et al*, 1996). SRC-1 has both intrinsic HAT activity and can recruit other HATs such as p300/CBP-associate factor (PCAF) for increased chromatin accessibility (Spencer *et al*, 1997). The interaction between PR and its co-regulators is thought to stabilise the pre-initiation complex, allowing for transcription factor binding and the recruitment of RNA polymerases to activate transcription (Klein-Hitpass *et al,* 1990).

The ATAC-seq data showed that the upon treatment with cAMP/MPA, the chromatin conformation around the PLCL-1 locus was in an open state, indicating increased chromatin accessibility, suggesting that cAMP/MPA recruited histone acetyltransferase to the PLCL-1 promoter site. Using genome browser/ENCODE/Factorbook motif; the ATAC-seq identified area was shown to be rich in transcription factor binding. Using ChIP-qPCR on this region, I showed that cAMP/MPA increased binding of transcription factors: CTCF, YY1 and ZNF143. CTCF and YY1 are both transcriptional activators and repressors, but ZNF143 is thought to be only a transcriptional activator. Both ZNF143 and YY1 interact with CTCF, and there being evidence showing that this interaction leads to transcriptional activation (Donohoe *et al*, 2007) (Bailey *et al*, 2015).

It is hypothesised that progesterone increases transcription of PLCL-1 because PLCL-1 acts an effector protein for progesterone's pro-quiescent function in the myometrium, and a loss of PLCL-1 during labour leads to a loss in quiescence and activation of contractions. PLCL-1 acts as an effector protein of progesterone using different mechanisms, with the key mechanism being the ability of PLCL-1 to reduce $[Ca^{2+}]_i$ in MSMCs. Regulation of $[Ca^{2+}]_i$ by PLCL-1, may be the reason why PLCL-1 is able to modulate changes in the transcriptome, in particular changes in genes involved in quiescence or parturition. RNA-sequencing showed that PLCL-1 acts as an effector of progesterone by decreasing expression of the pro-contractile phosphodiesterases, leading to the inhibition of cAMP metabolism.

PLCL-1 has multiple biological functions due to its nature as a scaffold protein, with numerous binding proteins. PLCL-1 is grouped within a rare cohort of proteins that can bind two of the most ubiquitous phosphatases: PP2a and PP1 (Sugiyama *et al*, 2013). PLCL-1 binds to and inhibits PP1, but when phosphorylated by PKA, it releases PP1 but has a stronger binding affinity for PP2a. PP1 and PP2a have a vast number of cellular substrates, leading to either activation or inhibition of these substrates. Therefore, through the modulation of PP1 and PP2a, PLCL-1 is indirectly able to regulate a wide repertoire of proteins, which are involved in different biological functions including quiescence and parturition. In addition, PLCL-1 binds to and regulates Akt, which also has a vast number of cellular substrates. Through the binding of PP1, PP2a and Akt; PLCL-1 is able affect different molecular pathways and

cascades, which can explain the multi-functional nature of PLCL-1. Furthermore, PLCL-1 may have a physical function, by sequestering IP_3 from binding to IP_3 receptors on the ER and releasing intracellular calcium in MSMCs. The multi-functional nature of PLCL-1 and its diverse binding partners, categorises it as a very fascinating protein. The loss of PLCL-1 in the labouring myometrium leads to a loss in the pro-quiescent functions of PLCL-1, making PLCL-1 a viable future diagnostic biomarker for premature labour (Figure 7.4).

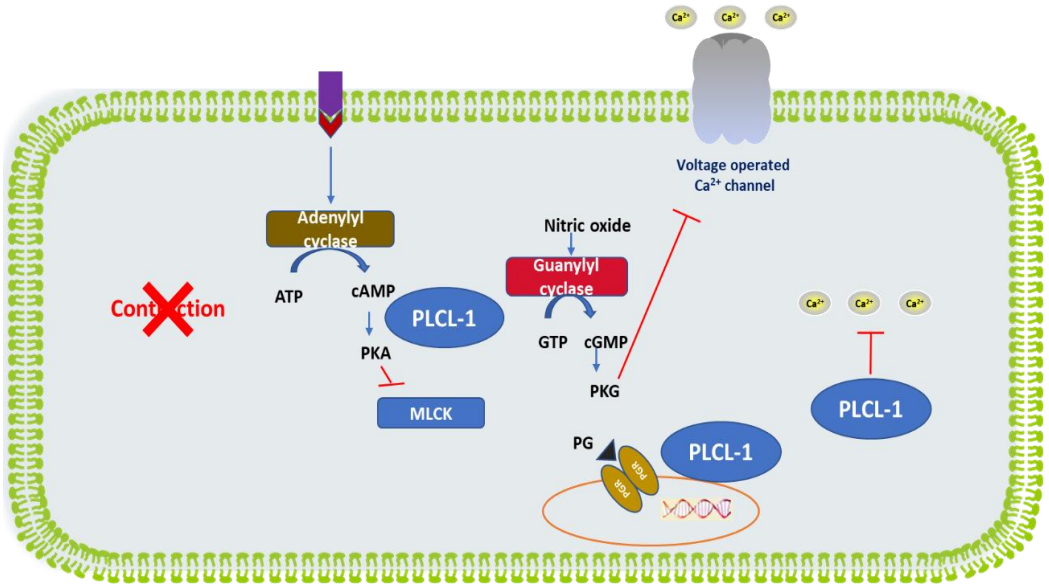

Figure 7.4- ***Proposed Role of PLCL-1 in the Maintenance of Uterine Quiescence During Human Pregnancy.*** PLCL-1 employs multiple mechanisms to inhibit myometrial contractions. PLCL-1 acts as an effector protein of progesterone signalling, it affects the transcriptome by inhibiting phosphodiesterase's, which metabolise the pro-quiescent cyclic nucleotides (cAMP and cGMP). PLCL-1 also reduces intracellular calcium concentrations.

7.5- Limitations and Future Direction

One of the main weaknesses in this thesis is the oxytocin treatment duration for the RNA sequencing samples. Only one gene oxytocin responsive gene (COX2) was measured, when deciding what the optimal oxytocin treatment duration was. Ideally, more oxytocin responsive genes should have been investigated to ensure that treatment duration chosen was in the maximal response window. Another limitation is that the data obtained from the ATAC-seq was from HESC and not MSMCs. Albeit, the data did show increased chromatin accessibility around the PLCL-1 locus, upon treatment with MPA and other studies done in numerous different cell types showed that the target region identified from the ATAC-seq data was rich in transcription factor binding. The target region identified also showed that MPA increased transcription factor binding in MSMCs (Chapter 6).

The blood samples did show that PLCL-1 is measurable from blood samples. A future direction would be to compare changes in PLCL-1 levels in the blood, between term women not in labour and term women in labour. This would show whether PLCL-1 is downregulated in blood during labour and thus if it is a potential preterm birth biomarker.

Another area of future work with PLCL-1 is to delete the ATAC-seq identified region using CRISPR/cas 9 and thereafter investigate if progesterone is still able to upregulate PLCL-1. The deletion would show that progesterone recruits co-activators

only to the ATAC-seq identified region in PLCL-1 promoter to upregulate expression of PLCL-1. Additionally, another area of future work could be investigating the effect of PLCL-1 on heat shock proteins.

7.6- Conclusion

In conclusion this thesis shows significant evidence that PLCL-1 is reduced in the labouring myometrium because it functions as a pro-quiescent protein. Progesterone upregulates transcription of PLCL-1 by increasing transcription factor binding to the PLCL-1 promoter. Subsequently, PLCL-1 acts as an effector protein for progesterone by inhibiting the release of calcium from intracellular stores and by maintaining baseline cAMP concentrations. The multiple mechanisms by which PLCL-1 functions in the myometrium, shows the complexity of pregnancy and the gene redundancy found not only in parturition but also in quiescence. Any future diagnostic test for preterm birth will have to take into consideration the multiple molecular mechanisms that maintain uterine quiescence, and thus will have to test for multiple biochemical markers. PLCL-1 could be one of the biochemical markers that is tested, if a method is established for testing localised PLCL-1 levels. Therefore, any further work on PLCL-1 will have to be on creating a diagnostic test that safely and accurately measures PLCL-1 levels in the female reproductive tract.

Appendices

Appendix 1 – Top Genes Contributing to PC9 Clustering, from their Loadings and Genes Up or Downregulated in all Patients

Gene Symbol	Gene Name
PLCL1	phospholipase C like 1
TBX5-AS1	TBX5 antisense RNA 1
LOC155060	AI894139 pseudogene
LOC101927701	long intergenic non-protein coding RNA 1812
PLA2G3	phospholipase A2 group III
LOC102724828	39S ribosomal protein L23, mitochondrial
PWAR5	Prader Willi/Angelman region RNA 5
SKOR1	SKI family transcriptional corepressor 1
BCL2A1	BCL2 related protein A1
WNT2	Wnt family member 2
LDB3	LIM domain binding 3
PDE7B	phosphodiesterase 7B
PRKG2	protein kinase cGMP-dependent 2
SULT1A1	sulfotransferase family 1A member 1
DHRS2	dehydrogenase/reductase 2
TMC4	transmembrane channel like 4
C21orf33	glutamine amidotransferase like class 1 domain containing 3A
SPDYA	speedy/RINGO cell cycle regulator family member A
PCDHB6	protocadherin beta 6
STX11	syntaxin 11
DDTL	D-dopachrome tautomerase like
CRYGS	crystallin gamma S
PAQR5	progestin and adipoQ receptor family member 5
CA9	carbonic anhydrase 9
UCA1	urothelial cancer associated 1
MYH1	myosin heavy chain 1
FCHO1	FCH domain only 1
NPB	neuropeptide B
SORCS3	sortilin related VPS10 domain containing receptor 3
RASL11B	RAS like family 11 member B
CYP19A1	cytochrome P450 family 19 subfamily A member 1
PARD6G-AS1	PARD6G antisense RNA 1
SYT14	synaptotagmin 14
COLEC10	collectin subfamily member 10
DACT3-AS1	DACT3 antisense RNA 1
GOLGA2P5	GOLGA2 pseudogene 5
LOC101929295	uncharacterized LOC101929295
HERC2P3	hect domain and RLD 2 pseudogene 3
TMEM9B-AS1	TMEM9B antisense RNA 1
SMG7-AS1	SMG7 antisense RNA 1
KCNE5	potassium voltage-gated channel subfamily E regulatory subunit 5

RN7SL2	RNA component of signal recognition particle 7SL2
KCNJ15	potassium voltage-gated channel subfamily J member 15
ESPNL	espin like
WNT7B	Wnt family member 7B
ZNF835	zinc finger protein 835
LOC100288846	uncharacterized LOC100288846
TLE6	TLE family member 6, subcortical maternal complex member
RGS22	regulator of G protein signaling 22
BMS1P5	BMS1 pseudogene 1
IRX3	iroquois homeobox 3
B3GALT5	beta-1,3-galactosyltransferase 5
THEGL	theg spermatid protein like
HOXD11	homeobox D11
EPHA7	EPH receptor A7
CATSPER3	cation channel sperm associated 3
FBXW9	F-box and WD repeat domain containing 9
CD40	CD40 molecule
FBXO16	F-box protein 16
PI16	peptidase inhibitor 16
ZNF572	zinc finger protein 572
FGF10	fibroblast growth factor 10
BEX5	brain expressed X-linked 5
PDE2A	phosphodiesterase 2A
SAMD5	sterile alpha motif domain containing 5
PDE3A	phosphodiesterase 3A
PHKG1	phosphorylase kinase catalytic subunit gamma 1
MMP7	matrix metallopeptidase 7

Appendix 2 - Down Regulated Genes: Transcript Per Million-Fold Change of PC9 Genes due to PLCL-1 Knockdown

GENE SYMBOL	GENE NAME	FOLD CHANGE
C21ORF33	glutamine amidotransferase like class 1 domain containing 3A	0.24475
MYH1	myosin heavy chain 1	0.26215
BEX5	brain expressed X-linked 5	0.27297
LDB3	LIM domain binding 3	0.29048
HERC2P3	hect domain and RLD 2 pseudogene 3	0.41867
DDTL	D-dopachrome tautomerase like	0.45070
SKOR1	SKI family transcriptional corepressor 1	0.45378
ZNF835	zinc finger protein 835	0.47439
PLCL1	phospholipase C like 1	0.49388
CA9	carbonic anhydrase 9	0.50013

RASL11B	RAS like family 11 member B	0.55213
THEGL	theg spermatid protein like	0.56395
WNT7B	Wnt family member 7B	0.57202
EFHD1	EF-hand domain family member D1	0.61906
GOLGA2P5	GOLGA2 pseudogene 5	0.65510
WNT2	Wnt family member 2	0.69976
LOC100288846	uncharacterized LOC100288846	0.70045
CACNA1H	calcium voltage-gated channel subunit alpha1 H	0.70238
LOC155060	AI894139 pseudogene	0.70667
PI16	peptidase inhibitor 16	0.71363
CRYGS	crystallin gamma S	0.71999
SPDYA	speedy/RINGO cell cycle regulator family member A	0.74702
KCNE5	potassium voltage-gated channel subfamily E regulatory subunit 5	0.77351
B3GALT5	beta-1,3-galactosyltransferase 5	0.77747
LOC102724828	39S ribosomal protein L23, mitochondrial	0.78078
NPB	neuropeptide B	0.78152
WNT5A	Wnt family member 5A	0.78360
SULT1A1	sulfotransferase family 1A member 1	0.84328
PDE3A	phosphodiesterase 3A	0.85066
PLA2G3	phospholipase A2 group III	0.86027
HOXD11	homeobox D11	0.86765
SAMD5	sterile alpha motif domain containing 5	0.87978
GRK5	G protein-coupled receptor kinase 5	0.88091
SYT14	synaptotagmin 14	0.89025
STX11	syntaxin 11	0.89994
EPHA7	EPH receptor A7	0.90039
FBXW9	F-box and WD repeat domain containing 9	0.91122
IRX3	iroquois homeobox 3	0.95849

Appendix 3 - Up Regulated Genes: Transcript Per Million Fold Change of PC9 Genes due to PLCL-1 Knockdown

GENE SYMBOL	GENE NAME	FOLD CHANGE
SORCS3	sortilin related VPS10 domain containing receptor 3	3.86272
LOC101927701	long intergenic non-protein coding RNA 1812	3.61847
FGF10	fibroblast growth factor 10	3.31439
SMG7-AS1	SMG7 antisense RNA 1	3.03501
BCL2A1	BCL2 related protein A1	2.81379
UCA1	urothelial cancer associated 1	2.50498
TBX5-AS1	TBX5 antisense RNA 1	2.35606
DACT3-AS1	DACT3 antisense RNA 1	2.26310

MMP7	matrix metallopeptidase 7	2.17142
PDE2A	phosphodiesterase 2A	2.04842
CATSPER3	cation channel sperm associated 3	2.03671
FBXO16	F-box protein 16	1.98600
TMEM9B-AS1	TMEM9B antisense RNA 1	1.95586
PRKG2	protein kinase cGMP-dependent 2	1.84421
LOC101929295	uncharacterized LOC101929295	1.75409
PCDHB6	protocadherin beta 6	1.72971
TMC4	transmembrane channel like 4	1.61407
BMS1P5	BMS1 pseudogene 1	1.60250
CYP19A1	cytochrome P450 family 19 subfamily A member 1	1.59154
RGS22	regulator of G protein signaling 22	1.55308
PWAR5	Prader Willi/Angelman region RNA 5	1.49462
PARD6G-AS1	PARD6G antisense RNA 1	1.48373
ZNF572	zinc finger protein 572	1.46641
PDE7B	phosphodiesterase 7B	1.46559
RN7SL2	RNA component of signal recognition particle 7SL2	1.37856
FCHO1	FCH domain only 1	1.37470
PAQR5	progestin and adipoQ receptor family member 5	1.35338
TLE6	TLE family member 6, subcortical maternal complex member	1.33066
KCNJ15	potassium voltage-gated channel subfamily J member 15	1.30830
ESPNL	espin like	1.28572
CD40	CD40 molecule	1.27151
DHRS2	dehydrogenase/reductase 2	1.18397
COLEC10	collectin subfamily member 10	1.13287
PHKG1	phosphorylase kinase catalytic subunit gamma 1	1.01668

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